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The function of human MOB2 in cell spreading in fibrosarcoma cells

ABSTRACT

Cell spreading is an initial mechanism for cell migration which plays a vital role in cancer development. Cell spreading has been shown to act as one of the key regulating steps between static and metastatic transition of a cancer cell. Hence, by identifying regulatory networks controlling cell spreading, it may provide valuable information and therapeutic strategies for preventing tumor metastasis. Both cell spreading and cell migration involve actin polymerization at the leading edge of plasma membrane follow by cell retraction at the rear end of cells. The molecular mechanisms in regulating cell spreading and cell migration have been extensively studied but remain unclear. Studies from yeast, *Drosophila* to mammalian cells have shown that MOB2 protein plays an important role in controlling the cell morphology changes by affecting cell polarity and rearrangement of actin cytoskeleton. Currently there is no research done to study the function of Mob2 in cell spreading and cell migration. In this study, we identified hMOB2 protein which plays a significant role in promoting cell spreading in HT1080 human fibrosarcoma cells. Our results showed that hMOB2 was detected at the leading edge of migrating HT1080 human fibrosarcoma cell. To study whether hMOB2 was involved in cell motility, we downregulated hMOB2 expression using RNA interference and found that cell spreading was delayed in HT1080 cells. In addition, we observed that overexpression of hMOB2 enhanced cell spreading in HT1080 cells and enhanced its accumulation at the leading edge. Furthermore, to determine the possible functional domain in cell motility, we successfully generated A107G, Y110A point mutated hMOB2

stable cell lines. Over-expressed point mutated hMOB2 expression delayed cell spreading and suppressed its accumulation at the leading edge. These observations suggested that hMOB2 affects cell spreading by regulating its expression at leading edge. No significant difference was observed in the migration rate between the different HT1080 cell populations when the percentage of gap closure was determined. However, over-expressed wild type hMOB2 induced broad lamellipodial structures and moved as a coherent group when compared with parent cells. These studies provided additional information on the molecular mechanisms which control cell spreading.

人類 MOB2 蛋白在纖維肉瘤細胞中扮演細胞延展的功能探討

摘要

細胞移動的初步機制，必須在基材上進行延展。細胞延展在癌細胞轉移中扮演着一個關鍵性的調控步驟。因此透過調控細胞延展的研究可以提供防止細胞轉移的治療策略。細胞延展和爬行都涉及actin聚合在細胞膜的前緣和尾部抽離。調節細胞延展和爬行雖已被廣泛研究，但詳細分子機制仍不清楚。根據以往的研究，Mob2蛋白會藉由參與actin細胞骨架的重新排列進而影響神經纖維生長。此外，Mob2在酵母菌的極性生成和果蠅感光細胞的發育過程中也影響actin細胞骨架。但是目前沒有研究指出Mob2是否會影響細胞延展和細胞爬行功能。在本研究中，我們發現人類纖維腫瘤(HT1080) 細胞展延需要hMOB2蛋白參與。免疫細胞染色結果顯示，人類纖維腫瘤(HT1080) 細胞爬行時，hMOB2會分佈在爬行的細胞前緣。進一步探討hMOB2是否參與細胞移動，我們利用shRNA的方式降低細胞內hMob2蛋白的表現觀察細胞延展和細胞爬行的情況，hMOB2在人類纖維腫瘤(HT1080) 細胞會延遲細胞展延。除此之外，過量表達野生型hMOB2蛋白會促進hMOB2累積在細胞

的前緣和增強細胞延展能力。為了更進一步瞭解hMOB2在細胞移動時的功能性區域，我們建立了持續表達突變型（A107G、Y110A）的hMOB2蛋白。結果顯示突變型的hMOB2蛋白會影響hMOB2蛋白在細胞前緣的累積及延遲細胞延展的能力。綜合以上結果，推測hMOB2蛋白會透過影響其表現在細胞前緣，來調控細胞展延。然而在人類纖維腫瘤細胞(HT1080)移動的速率，降低細胞內hMob2，表現野生型和突變型的細胞株中，細胞移動的速率並沒有顯著的差異。過量表達野生型hMOB2蛋白會形成較多的板狀偽足結構，並且會以群體方式來移動。此研究結果提供對細胞延展的分子機制有更進一步的了解。

1.0 INTRODUCTION

1.1 Cell spreading

Cell spread on a substratum is an initial mechanism for cell adhesion, proliferation and migration, which mutually plays a vital role in wound repair, immunity and cancer development. When cancer cells travel away from primary site to another parts of the body, grow and form colony tumors, this process is called metastasis. Most cancer patients died due to metastatic cancer. Cell spreading is one of the key regulating steps between the static and metastatic transition of a cancer cell (Shen *et al.*, 2007). Hence, by identifying regulatory networks controlling cell spreading, it may provide valuable information and therapeutic strategies for preventing tumor metastasis.

Cell spreading involves deformation of plasma membrane and followed by the formation of cell-substrate attachments. It is divided into passive and active events. In early spreading, a passive event, cells do not require metabolic energy to expand but involved binding and attachment to substrate. However, in later spreading, the active processes of actin polymerized at the leading edge of the cell membrane. It would then extend the cell, leading to cell flattening and increase in overall surface area. The cell then changes from spheroid shape to nearly discoid conformation. In much later stage of cell spreading, myosin contraction create polarized cells and generate locomotion force promoting cell to migrate (Mc Grath, 2007; Dubin-Thaler, 2004). Since cell spread on a substratum will eventually increase its overall surface area, therefore, cell spreading is commonly characterized or evaluated by its overall surface area occupied by the spread

cell on a substratum (Brugmans *et al.*, 1982; Tsumura *et al.*, 2005).

1.2 Cell Migration

After the cell is attached to the substratum and spread, myosin contraction will eventually take place, leading to cytoskeleton rearrangement and causing cell to enter cell migration steps. Cell migration involves extension, adhesion, translocation, de-adhesion and endocytic recycling. Cell movement begins with extension of one or more lamellipodia from leading edge of the cells. Some lamellipodia then adhere to the substratum by focal adhesion. The bulk of cytoplasm in cell body flows forward due to contraction at the rear of cell. However, the trailing edge still remains attached to the substratum until the tail end eventually detaches and retracts into the cell body. During this process, cytoskeleton-based cycle, endocytic cycle and integrins at rear end of the cell will be transport to the cell front for re-use in making new adhesion (Lodish *et al.*, 2008). Cell migration study is valuable for cancer cells study, embryogenesis and immune response.

1.3 Signal involves in actin polymerization

Actin polymerization is actively involved in cell spreading and cell migration. The members of Rho GTPase family have been shown to regulate actin dynamics, which involved in cellular processes such as cell spreading, migration, cytokinesis, and morphogenesis. It is active in the GTP-bound state and inactive when bound to GDP.

Each small GTPase family molecules control a signal transduction pathway linking membrane receptors to assembly actin cytoskeleton. Three members of the family have been studied in a great deal, for instance, Cdc42 activates formation of filopodia, Rac activates the formation of lamellipodia, and Rho activates the formation of stress fiber and plays an important role in cell contraction (Hall, 2005).

In cell spreading process, it has been shown that inhibition of RhoA pathway facilitates Rac and Cdc42 activation and lead to filopodia and lamellipodia formation at cell cortex to promote cell spreading (Flevaris *et al.*, 2007). Membrane-based integrin has been shown to interact with Extracellular Matrix (ECM) to initiate a complex cascade of signaling events for a cell to spread. Flevaris and colleagues further proposed c-Src binding with C-terminal domain of integrin β_3 will inhibit RhoA-dependent cell retraction; as a result promote actin polymerization at leading edge of cell to induce cell spreading (2007).

In a migrating cell, Cdc42 activating at cell front leading to filopodia formation followed by Rac activation forming lamellipodia at cell front. These structures generate locomotion force, in turn activate Rho, forming stress fiber leads to interaction of myosin with actin filaments induces retraction at the trailing edge (Lodish *et al.* 2008). This phenomenon coordinates reorganization of actin cytoskeleton and permits the cell to move forward.

Although the molecular mechanisms in regulating cell spreading and cell migration have been extensively studied, however, there are other molecules that participated in cell spreading and cell migration which are still remain unclear and yet to be investigated.

1.4 MOB protein

Mps One Binder (Mob) protein was first suggested by Luca and Winey in year 1998 from yeast. Mob proteins shared high sequence similarity and are characteristic of conserved Mob1_phocein domain that shared among them. Phocein is a multi-protein complex and shown to interact with NDPK, Eps 15, dynamin and proteins of striatin family. Phocein is functionally involved in signaling, vesicular trafficking and endocytosis (Baillat *et al.*, 2002; Bailly and Castets, 2007). Mob proteins are highly conserved from yeast to human. Two distinct Mob proteins have been indentified in yeasts, such as Mob1 and Mob2, while an expansion in metazoans, Mob protein gives rise to six MOB proteins in humans and mouse such as MOB1A, MOB1B, MOB2, MOB3A, MOB3B, and MOB3C and four in *Drosophila melanogaster* such as Dmob1, Dmob2, Dmob3 and Dmob4.

Mob proteins have been reported generally would interact with family members of Nuclear Dbf2-related (NDR) kinase, act as co-activators of NDR from yeast (Komarnitsky *et al.*, 1998; Hou *et al.*, 2004; Weiss *et al.*, 2002; Hou *et al.*, 2003), *Drosophila*, (Hergovich *et al.*, 2006) to human (Hergovich *et al.*, 2008). Member of NDR is a subclass of AGC-type serine/threonine protein kinases. They participated in controlling important cellular processes such as mitotic exit, cytokinesis, cell proliferation and morphogenesis, and apoptosis. The acidic surface of Mob protein was predicted as a region which interacts with NDR family protein through electrostatic interaction (Hergovich *et al.*, 2008).

1.4.1 MOB structure

Mob proteins as mentioned above are characteristic of conserved Mob1_phocein domain, with around 180 amino acid residues in length. Mob proteins are divided into different groups due to exon-intron structures (Ye *et al.*, 2009). However, they appeared to share three major structural features. 1) MOB proteins have highly conserved zinc finger structure responsible for zinc binding. 2) One side of MOB proteins contain an acidic flat surface, predicted as a critical side to interact with its partners. 3) MOB family proteins are small and contain no other known structural motif (Ye *et al.*, 2009). Since MOB proteins contained conserved zinc finger structure, Ye and colleagues predicted that zinc finger might be a critical structural feature for MOB family proteins. Based on human MOB1A protein X-ray crystal structure resolved by Stavridi and colleagues in year 2003, they revealed that Cys_2His_2 form a zinc binding site holding a zinc atom in the middle and participate in four-helix bundle core structure folding in hMOB1A. Additional studies showed that the removal of zinc using EDTA will contribute to protein aggregation and affect its thermal stability (Ponchon *et al.*, 2004). Ye and colleagues then used structure of hMOB1 as a template, mapped the amino acids of each group of MOB proteins (Ye *et al.*, 2009). They predicted besides Cys_2His_2 as mentioned above, Ala 111 and Tyr 114 are another two amino acid conserved in each group of MOB proteins which spatially close to zinc finger motif and facing inward to the core of helix bundle. In turn, it may contribute to the protein stability by hydrophobic interactions (Ye *et al.*, 2009).

1.4.2 Mob2 in yeast study

Mob2 protein was first suggested by Luca and Winey in year 1998 from budding yeast, *Saccharomyces cerevisia*. Mob2 interacts with Cbk1 kinase (second NDR/LATS kinase in *S. cerevisiae*) to regulate daughter-specific genetic programs and induce asymmetric cell fate in budding yeast (Lerner *et al.*, 2001). In addition, Hou and colleagues also found that in fission yeast, *Saccharomyces pombe*, Mob2 formed complex with Orb6 (second NDR/LATS kinase in *S. pombe*) to participate in polarized cell growth and cell cycle control (Hou *et al.*, 2003). Authors demonstrated that deletion of mob2 in yeast is lethal and causes cells become spherical with depolarized actin and microtubule cytoskeletons. These eventually affect cell morphology and re-organization of actin cytoskeleton and microtubules. Moreover, decrease of Mob2 protein will result in defected bipolar growth (Hou *et al.*, 2003). Together, the above results concluded that Mob2 play an important role in regulating cell morphogenesis and determine cell polarity.

1.4.3 Dmob2 in *Drosophila* study

Drosophila mob2 (*Dmob2*) has been shown to form complexes with Tricornered (Trc) kinases (one of the NDR/LATS kinase in *Drosophila*) and plays a role in wing hair morphogenesis (He *et al*. 2005). Recently, dMOB2 has been reported to participate in photoreceptor development and contribute to rhabdomere formation (Liu *et al.*, 2009). During rhabdomeres formation, cell polarity determination and actin cytoskeleton organization are crucial to affect rhabdomeres which appear as oval and localizes

specifically in the center of apical membrane (Pham *et al.*, 2008). In down-regulated Dmob2 expression flies, the shape of rhabdomeres appeared irregular. Some rhabdomeres widely expanded and some were missing (Liu *et al.*, 2009). Based on the above observation, Liu further suggested that Dmob2 affect rhabdomeres formation involve in cell polarity regulation and actin cytoskeleton organization (Liu *et al.*, 2009).

1.4.4 Mob2 in mammalian cells study

In mammalian study, Mob2 has been shown to recruit NDR to plasma membrane allowing NDR to interact with its upstream kinase (Hergovich *et al.*, 2005). Recent findings revealed that hMOB2 competes with hMOB1 to form complex with NDR, it inhibits the phosphorylation of NDR and blocks its kinase activation (Kohler *et al.*, 2010). Kohler and colleagues further showed that overexpression hMOB2 delay with NDR kinase apoptotic function and block NDR functions in centrosome overduplication during S-phase (Kohler *et al.*, 2010). However, detailed studies are yet to be elucidated.

Besides that, in Lin's study, mouse Mob2 protein has been shown to promote neurite formation in mammalian Neuro2A cells (Lin *et al.*, 2011). Overexpression of Mob2 in N2A cell promoted neurite formation whereas down-regulated Mob2 suppressed neurite sprouting in N2A cells (Lin *et al.*, 2011). Similar results also shown in Hu's studies (unpublished data), over-expressed Mob2 in NIH3T3 cells also causes neurite formation. In addition, Lin and colleagues further showed that in down-regulated Mob2 cells, actin appeared as fragments in cytoplasm (Lin *et al.*, 2011). However, in control-shRNA treated N2A cells, actin filaments present occupied the whole cytoplasm and

formed a tight band near subcortical region with extended several filopodia from the cell membrane (Lin *et al.,* 2011). Since actin filament and microtubules actively involved in initial neurite sprouting, these findings suggested that Mob2 may trigger the rearrangement of actin cytoskeleton and initiate neurite formation.

1.5 Specific aims on my studies

Studies from yeast, *Drosophila* to mammalian cells have shown that MOB2 protein play an important role in controlling cell morphological changes by affecting cell polarity and rearrangement of actin cytoskeleton. In addition, actin polymerization and rearrangement of actin cytoskeleton have been shown to play an equally important role in cell spreading and cell migration. Currently there is no research to observe the function of MOB2 in cell spreading and cell migration. We wondered what role, if any, MOB2 protein plays in cell spreading and cell migration.

In this study, we investigated whether the MOB2 protein in human (also known as hMOB2 in further text) plays a similar role in organizing actin filaments and regulating cell spreading and cell migration. We used HT1080 fibrosarcoma cells, a malignant tumor derived from connective tissue and characterized as immature proliferating fibroblasts, to study how human MOB2 participated in cell spreading and cell migration. Immunocytochemical study indicated that hMOB2 located at cytoplasm and co-localized with F-actin at the leading edge of plasma membrane. Using RNA interference to knockdown hMOB2 expression, we found interfered cell spreading whereas overexpression of hMOB2 enhanced cell spreading in HT1080 cells. Similar

result was also observed in point-mutated hMOB2 cells where cell spreading was affected. Our study showed that hMOB2 has potential to regulate cell spreading. Although the motility of HT1080 cells is not impacted by hMOB2 expression, collective migration phenotype (qualitative changes) has been observed in migration of over-expressed hMOB2 cells. Taken together, we hoped to provide additional information on the molecular mechanism controlling cell spreading.

2.0 MATERIALS AND METHODS

2.1 Culture HT1080 cells

HT1080 fibrosarcoma cells, as well as over-expressed hMOB2, point-mutated hMOB2 and shRNAs treated HT1080 cells were grown in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% fetal bovine serum (FBS), 2mM L-Glutamine, 1% penicillin/streptomycin (P/S), and 1% non-essential amino acids maintained at 37^{o}C in a humidified atmosphere with 5% CO_2.

2.2 Mob2 RNA interference

To knockdown the endogenous hMOB2 expression, the pLKO.1 short hairpin RNA (shRNA) plasmids encoding shRNA with scrambled sequences (Clone ID: TRCN0000072179) or sequences targeting human mob2 (hmob2-shRNA1, Clone ID: TRCN0000166012, corresponding coding sequence: GCGAGATTGACCTTAACGAGT; hmob2-shRNA2, Clone ID: TRCN0000165158, corresponding coding sequence: GATCACCGACTTCCAGTTCAA were purchased from the National RNAi Core Facility, Taiwan.

2.3 Infection of HT1080 cells

shRNA with scrambled sequences and hmob2-shRNAs were sent to National RNAi Core Facility individually packed into pVSV-G to generate VSV-G-pseudotyped lentiviral

particles containing an expression construct. Pseudotyped lentiviral particles were infected to HT1080 cells. A day before infection, $3x10^5$ cells were seeded on 3.5cm culture dishes. An M.O.I of 3 was used in each infection in 1mL media containing 8μg/mL polybrene. 24 hours post-infection, puromycin was added into the medium at the final concentration of 0.6μg/mL to select the infected cells. Cells were harvested at 4 days postinfection. To monitor the efficacy of hmob2-shRNAs, the expression of hMOB2 in HT1080 stable infection was analyzed by Western Blotting and probed with anti-MOB2 antiserum.

2.4 Construction of plasmids

2.4.1 RNA extraction

The day before RNA isolation, $5x10^6$ of HT1080 cells were seeded in 3.5cm culture dishes. RNA was isolated from HT1080 cells using 1mL of TRIzol reagent (Invitrogen Life Tech), pipetted for several times. Suspension was transferred to new microcentrifuge tube and incubated for 5 minutes at room temperature. 0.2mL chloroform was added to purify RNA and shaked for 15 seconds before being centrifuged at 12000rpm for 15 minutes. Upper aqueous phase was removed to a new eppendorf tube, 0.5mL isopropyl alcohol was added and incubated at room temperature for 10 minutes to precipitate RNA before centrifuge again at 12000rpm for 10 minutes at 2-8^oC. RNA pellet at bottom was washed with 1mL of 75% ethanol and followed by 7500rpm centrifugation for 5 minutes at 2-8^oC. RNA pellet then allowed to dry for 5 minutes in 65^oC incubator and dissolved in 12μl DEPC water. 2μl was taken for RNA detection using 1% of agarose performed gel

electrophoreses for 10 minutes. 1µl of total RNA was taken to detect RNA concentration.

2.4.2 Reverse transcription-Polymerase Chain Reaction

Complementary DNA was synthesized from 3µg of total RNA using SuperScript® II Reverse Transcriptase from Invitrogen Life Tech. Total volume of 20µl from the reaction; 2µl of synthesized complementary DNA was used as template to construct expression vectors. Wild type hMOB2 forward primer has the sequence of **gccgccggatccatggactggctcatggggaa** contained BamH*I* digestion site; meanwhile the reverse primer has the sequence of **gccgccctcgagcgtctctccttcacgtggttctgtg** contained Xho*I* digestion site. PCR-directed mutagenesis was used to achieve the desired mutations. The primer for A107G, Y110A point mutation hMOB2 has forward primer with the sequence of **acggcctgtgggcccg** and reverse primer has **cgggcccacaggccgt** sequence coding the intended A107G, Y110A mutation. A107G, Y110A point mutation hMOB2 obtained with intended mutation site was used as template for second round of PCR using wild type hMOB2 forward and reverse primers to obtain full length of A107G, Y110A point mutation hMOB2. PCR was carried out in 50µl reactions with final concentration of 1x PCR buffer containing $MgCl_2$ (Invitrogen Life Tech), 0.25mM dNTPs (BERTEC), 10µM of each forward and reverse primer pair, and 2.5U Taq DNA polymerase (Invitrogen Life Tech). PCR was performed in Minicycler™ PCR machine for an initial denaturation step of 5 minutes at 95°C followed by 30 cycles, each consisting of 95°C denaturation for 30 seconds, 56°C annealing for 1 minute and 72°C extension for 30 seconds. A 7 minutes final extension was performed at 72°C and the PCR products were held indefinitely at 4°C. PCR products were electrophoresed on 1% agarose gel. PCR

products with the accurate amplified DNA fragments were purified using Gel/PCR DNA fragments extraction kit (Geneaid).

2.4.3 Restriction endonucleases

Wild type hMOB2 and A107G, Y110A point-mutated hMOB2 DNA fragments obtained were individually cut with Bam*HI* and Xho*I* digestion site. Restriction enzyme was carried out in 50µl reactions with final concentration of 1x buffer (Bam*HI* buffer), 1x BSA, 1.5µl Bam*HI*, 1.5µl Xho*I*, DNA, and double distilled water toped up to 50µl. Reaction incubated at 37^{o}C for 5 hours. Effectiveness of digestion site was evaluate using 1% agarose gel electrophoreses and purified using Gel/PCR DNA fragments extraction kit (Geneaid).

2.4.4 Ligation

Wild type hMOB2 and A107G,Y110A point-mutated hMOB2 digested DNA fragments with sticky end were ligated to the pcDNA^{TM}3.1/myc-His.B expression vector to yield pcDNA-hMOB2 and pcDNA-A107G,Y110A point-mutated hMOB2. Ligation was carried out in ratio of vector versus insert, 3:1, performed at 16^{o}C, incubated overnight with 2µl T4 buffer (New England Biolabs), 1µl T4 ligase (New England Biolabs) and top up with distilled water to 20µl reactions.

2.4.5 Bacterial Transformation

10µl of ligase reaction were taken to mix with 80µl of competent cells (DH5α) to facilitate the take up of DNA into bacterial cell's membrane. DNA were forced into the

cells by incubated the cells and DNA on ice for 20 minutes, followed by heat-shock for 40 seconds at 42^{o}C water bath and put on ice to recover for 2 minutes. 800µl TB medium was added into the competent cells and shaken for 1.5 hours at 37^{o}C, 185rpm. Cells were then plated out on ampicillin containing LB plate and allowed to grow for 12-16 hours at 37^{o}C incubator.

2.4.6 Plasmid DNA extraction

Single colony from ampicillin containing LB plate was picked and amplified in 2 mL LB with ampicillin incubated overnight at 37^{o}C for 185rpm. Plasmid DNA then extracted using Plasmid Miniprep Kit (BioKit) and detected using 1% of agarose performed gel electrophoreses. Both constructs were verified by DNA sequencing before processing for experiment.

2.5 Transfection of HT1080 cells

Wild type hMOB2 and point-mutated hMOB2 constructs generated were individually transfected into HT1080 cells. Lipofectamine^{TM} and PLUS^{TM} reagents (Invitrogen Life Tech) were used for transfection. 2x10^{5} cells seeds in the morning and transfection performed in the evening with 1µg of DNA in 8µl PLUS^{TM} reagents in 100µl of transfection medium and 10µl of Lipofectamine^{TM} in 100µl of transfection medium. Each allowed incubate at RT for 15 minutes. Each reagent then mixed together became complex mixtures and allowed to stand at 45 minutes at room temperature before added into HT1080 cells contained 1mL DMEM complete. Medium was changed on the next

days and followed by G418 added to select the transfected cells at the final concentration of 1mg/mL. After transfection, the HT1080 cells were collected and processed for immunofluorescence and Western blotting. Cell clones were selected by growth in the presence of 1mg/mL G418 to establish over-expressed wild type hMOB2 and point-mutated hMOB2 stable cell lines.

2.6 Western Blot

Confluent cells were washed with 0.1M phosphate-buffered saline (PBS) before scrape to remove from plate surface in 1mL 0.1M PBS. Cells in suspension then centrifuged at 8000rpm for 5 minutes in 1.5mL eppendorf tube. Cells pellets were resuspended in 20µl of RSB cell lysis buffer contained 10mM HEPES, 10mM NaCl, 1.5mM MgCl2, 1m DTT. Solution was supplemented with 1 mM phenylmethylsulfonylfluoride, and protease inhibitor cocktail (10µg/mL leupeptin, 10µg/mL aprotinin). Cell lysates were incubated on ice for 30minutes followed by 1 hour incubation at -20^{o}C. After centrifugation at 12500 rpm for 30 minutes at 4^{o}C, protein concentration was determined by a Bio-Rad Protein Assay (Bio-Rad) using spectrophotometer (Bio-Rad Smart Spec^{TM} Plus). Proteins (20mg) were denatured in 4X SDS gel loading dye for 5 minutes at 95^{o}C and recovered on ice for 5 minutes. The proteins (12µl) were separated by 10% polyacrylamide gel. Gel with separated proteins then transferred to a Polyscreen® PVDF (polyvinylidene fluoride) transfer membrane (PerkinElmer) in stack form with membranes in Trans-blot$^{@}$SD Semi-Dry Transfer cell (BioRad), in condition of 20 voltages in 35 minutes. After transfer, non-specific binding on PVDF was blocked with 5% non-fat milk in 0.2% Triton-X100 in

PBS, incubated at 4^{o}C overnight. With constant shaking, the membrane were probed with designated primary antibody in 0.2% Triton-X100 in PBS in a seal-a-meal bag on a nutator for 2 hours at room temperature. Unbound primary antibody was washed away with 0.2% Triton-X100 in PBS. After three times washes with 0.2% Triton-X100 in PBS each time 10 minutes, the membranes were incubated in secondary antibody diluted in 0.2% Triton-X100 in PBS, reacted for 1 hour at room temperature. Unbound secondary antibody was washed away with 0.2% Triton-X100 in PBS, three times each incubated for 10 minutes. Immunodetection was performed using the LAS-1000 luminescent image analyzer detection system (Fujifilm).

Primary antibody: Rabbit anti-Mob2 (1:3000; Lin et al., 2011)

Mouse anti-tubulin (1:7000; Sigma-Aldrich)

Secondary antibody: Anti-rabbit-HRP (1:5000; Jackson ImmunoResearch Lab)

Anti-mouse-HRP (1:5000; Jackson ImmunoResearch Lab)

2.7 Immunofluorescence

Cells grown under cover slide were fixed with 4% paraformaldehyde in 0.1M PBS (pH7.4) for 20 minutes at room temperature. Fixed cells were washed with 0.1M PBS and then permeabilized with 0.2% Triton-X100 in PBS for 15 minutes. After three washed with 0.2% Triton-X100 in PBS and blocked with 10% FBS in 0.2% Triton-X100 in PBS, the cells were incubated with primary antibody and rhodaimine-phalloidin for F-actin (Sigma-Aldrich) at 4^{o}C overnight or at room temperate for 1.5 hours. After three washes with 0.2% Triton-X100 in PBS, cells were incubated with secondary antibody

included SYTOX® Green for nucleus (Invitrogen Life Tech) at room temperature (avoid from light expose) for 1 hour. After three washes with 0.2% Triton-X100 in PBS, cells were mounted in 10μl of mounting medium (80% Glycerol). Cells were examined on a Zeiss LSM510 confocal microscope (Zeiss, Germany).

Primary antibody: Rabbit anti-Mob2 (1:300; Lin et al., 2011)

Phalloidin (1:200; Sigma-Aldrich)

Secondary antibody: Donkey anti-Rabbit-Cy5 (1:200; Molecular Probes-Invitrogen)

Sytox (1:1200; Invitrogen Life Tech)

2.8 Wound healing assay

Cells were plated onto 3.5cm culture dishes at a saturation density of $1x10^6$ cells. Cells were then scratched manually with a pipette tip 24 hours after plating. The wounded regions were allowed to heal for defined time in DMEM complete. Images for these cells were observed by Axiovert200M phase-contrast microscopy (Carl Zeiss). Images were collected with a 10x objective 0, 3, 6, 10 hour after wounding. Images were quantitated using AxioVision Rel4.7 software by measuring the surface area remains between wound edges after one sample achieved 100% wound closure.

2.9 Cell spreading

For cell spreading assay, cells were suspended and replated on 3.5cm culture dishes. After 30 minutes, first hour and second hour incubation at 37^oC, cells were taken for capture

under Axiovert200M phase-contrast microscopy (Carl Zeiss). To obtain quantitative data of extent of cell spreading, the cell areas were calculated using AxioVision Rel4.7 software, and cells were categorized into three classes: cell area< 400 μm^2 (Class 1, round cells weakly adhered onto the substrate), 400 μm^2 < cell area < 800 μm^2 (Class 2, cells in the course of spreading with extended pseudopodia) and cell area > 800 μm^2 (Class 3, flat and well-spread cells with extended lamellipodia).

2.10 Cell proliferation assay and population doubling time

Cells were seeded at $4x10^4$ cells/well into 24-well plates in DMEM complete containing 10% FBS. After 24 hours incubation, cells were washed with 0.1M PBS follow by trypsin to resuspend the cells. 10µl of cell suspensions were added to 10µl of trypan blue, mixed well and 10µl of mixture were taken for cell count. Cells were counted under Hemocytometer. Cell concentration per ml (and the total number of cells) will be determined using the following calculations.

Cells per ml = the average count per square x the dilution factor x 10^4

Same procedure repeated to determine cell concentration after cells incubated for 48 hours, 72 hours and 96 hours. Experiments were performed in triplicate. Population doubling times of the different cell cultures were calculated using *Doubling Time Software v1.0.10* (http://www.doubling-time.com) (Roth, 2006).

3.0 RESULTS

3.1 hMOB2 expression in HT1080 cells

To identify whether Mob2 proteins are highly conserved among different groups, human and mouse Mob2 proteins were used to make alignments (Fig. 1). Although mouse Mob2 protein size was smaller than human MOB2 (also known as hMOB2) protein size, by 2 amino acids, yet both proteins alignments shown highly conserved with 91% identity. Since mouse Mob2 and human MOB2 are highly conserved, to study the possible function of hMOB2, we used mouse Mob2 antibody generated in Lin *et al.* study. Western blots demonstrated that the hMOB2 protein was detected using mouse Mob2 generated antibody (Fig. 2). We then used immunocytochemistry to determine the subcellular localization of hMOB2 protein in the fibrosacroma cells (HT1080). Immunocytochemistry reveal hMOB2 protein was mainly localized at the cytoplasm and at the leading edge of plasma membrane. The localization of hMOB2 detected at the leading edge of plasma membrane is similar to the position of lamellipodia, actin-dependent protrusion formed during cell spreading process. Together, these results suggest a potential function for the hMOB2 protein in cell spreading.

3.2 Downregulation of hMOB2 affects cell spreading

To study the function of hMOB2 in cell spreading, RNA interference was used to down-regulate hMOB2 expression. HT1080 cells were infected with two distinct

shRNAs that targeted the *hmob2* sequence and control scramble shRNAs, individually. After puromycin selection, the level of hMOB2 expression in HT1080 cells deceased to 70% and 69%, respectively, when compared to control shRNA (three experiments, *Student's t*-test, *** P<0.00) (Fig. 3). Immunocytochemical results demonstrated that down-regulated hMOB2 actin filaments stained by rhodamine-phalloidin occupied the whole cytoplasm with membrane ruffles. These were similar with the actin filaments localization in parent cells. Thus showed that down-regulated hMob2 does not affect the localization of actin cytoskeleton in HT1080 cells (Fig. 4). To assess the function of hMOB2 in cell motility, we performed two different functional assays.

We investigated the role of hMOB2 in cell spreading by determine the surface area of cells and categorized cells into three classes: cell area < 400 μm^2 (Class 1, round cells weakly adhered onto the substrate), 400 μm^2 < cell area < 800 μm^2 (Class 2, cells in the course of spreading with pseudopodia surround plasma membrane) and cell area > 800 μm^2 (Class 3, flat and well-spread cells with extended lamellipodia) (Fig. 5).

To study whether downregulation of hMOB2 affects cell spreading, HT1080 cells were individually infected with hmob2 and control shRNAs. The infected cells were suspended and replated on 3.5cm culture dishes. The cell images were captured at the first 30 minutes then followed by first hour and second hour to assay cell spreading. Figure 6A showed representative photographs (10x) of control cells. Cells surface areas were measured and separate into three classes. Quantitative analysis showed that after cells were replated for 30 minutes, control cells contained a high percentage fall in Class 1 and Class 2. When spreading time gradually increased, at second hour, cells observed shifted to Class 2 (Fig. 6B). Figure 6C and 6E represented two individual down-regulated

hMOB2 cells spread for 30 minutes, first hour and second hour after cells replated. Down-regulated hMOB2 cells observed presented in round and weakly spread condition compared with the control expressing cells. Quantitative analysis of hmob2-shRNA1 and hmob2-shRNA2 cells showed that overall Class 1 cells were observed throughout 2 hours after cell replated (Fig. 6D and F). Quantitative analysis of hmob2-shRNA1 and hmob2-shRNA2 cells cell spreading results at 30 minutes, first hour, and second hour were consistent. Quantitative analysis of control and 2 individual down-regulated hMOB2 at second hour after cells replated showed that Class 2 cells were decreased and that of Class 1 cells increased in down-regulated hMOB2 cells compared with control cells (Fig. 6G). Cells were quantified based on triplicate capture in each experiment repeated in 3 independent experiments. These results suggest that downregulation of hMOB2 expression delayed cell spreading of HT1080 cells.

3.3 Overexpression of hMOB2 promotes its accumulation at the leading edge of migrating cells and enhances cell spreading

We have shown that downregulation of hMOB2 expression affected cell spreading in HT1080 cells. We then test whether overexpression of hMOB2 promoted cell spreading in HT1080 cells by constructing wild type hMOB2 in pcDNA plasmid (Fig. 7). Protein sequence alignment showed wild type hMOB2 construct generated in pcDNA plasmid contained histidine-tag and myc-tag sequence. HT1080 cells were transfected with pcDNA-hMOB2, after G418 selection, several colonies of hMOB2 were selected to generate stable cells lines. We then used Western blot analysis to determine the

expression of hMOB2 in these stable cell lines. Results showed that HT1080 cells transfected with pcDNA-hMOB2 expressed larger size of the recombinant protein than endogenous protein (Fig. 8). The reason is probably due to recombinant protein containing Myc and His tag. To study the expression of over-expressed hMOB2 cells in HT1080, we stained cells with anti-MOB2 and rhodamine-phalloidin. Immunocytochemical results demonstrated that expression of pcDNA-hMOB2 in HT1080 cells enhanced hMOB2 accumulation at the leading edge of cells compared with parent cells with endogenous hMOB2 expression (Fig. 9). Besides that, extensive hMOB2 at the leading edge of cells were co-localized with F-actin stained by rhodamine-phalloidin.

When HT1080 parent cells and over-expressed hMOB2 cells replated on culture dish to determine cell spreading, over-expressed hMOB2 cells (Fig. 10C) spread faster than wild type (parent cells) (Fig. 10A) and induced broad lamellipodia structures at the leading edge of over-expressed hMOB2 cells. At 30 minutes after cell replating, the majority of Class 1 cells were observed in HT1080 parent cells (Fig. 10B). With the increased in time, at second hour, parent cell observed mainly at Class 2. However, at 30 minutes after cells replating, majority of Class 2 cells observed in over-expressed hMOB2 cells (Fig. 10D). At second hour, the percentage of Class 2 cells decreased and Class 3 cells increased in over-expressed hMOB2 cells. Cells were quantified based on triplicate capture in each experiment repeated in 3 independent experiments. Wild type and 2 individual over-expressed hMOB2 at second hour after cells replated were different (Fig. 10E). Class 2, actively spread cells mainly observed in wild type cells at 2 hours time whereas Class 3, majority observed in over-expressed of hMOB2. These results

suggested that over-expressed of hMOB2 expression enhanced cell spreading of HT1080 cells.

3.4 Structural conserved of Mob2 proteins

To further understand the structure-function relationship of hMOB2, we determined the possible functional domain of hMOB2. Previous laboratory study on mouse Mob2 protein which played a significant role in promoting neurite formation in N2A cells (Lin *et al.*, 2010) has shown that deletion region between 28 to 143 amino acids in mouse MOB2 gave no neurite outgrowth (Lin, unpublished data). Since mouse Mob2 protein and human MOB2 protein are highly conserved with 91% identity (Fig. 1), this raised the possibility that the functional domain in mouse Mob2 was the functional domain in human MOB2.

In addition, Stavridi *et al.* resolved X-ray crystal structure of human MOB1A protein depicted the highly conserved zinc finger structure around Mob1_phocein domain interacting with zinc atom. Since Human MOB proteins shared high sequence similarity and are characteristic of a conserved domain Mob1_phocein with around 174 amino acid residues in length (Fig. 11), this raised the possibility that the important zinc finger structure in hMOB1A was similar in hMOB2 as well and might contribute to an important role. More recently, Ye *et al.* had compared all groups of Mob proteins and uncovered six amino acids posted zinc finger structure. These six amino acids were Cys78, Cys83, Ala107, Tyr110, His157 and His 162. Importantly these six amino acids were conserved within human MOB proteins and among human and mouse Mob2

proteins.

To investigate the possible functional domain of hMOB2 in cell spreading, we combined above mentioned possible important sites in Mob2 protein, designed several deletion construct (Fig. 12). The deletion construct designed consists of point mutations at Cysteine78 and Cysteine83, point mutations at Alanine107 and Tyrosine110, big deletion within the same region as mouse Mob2, front region deletion, back region deletion and middle region deletion within the predicted site based on mouse Mob2 possible functional domain in neuritogenesis. To initiate the possible functional domain search, we decided to generate Ala107 and Tyr110 point-mutated hMOB2 construct. The study by Ye *et al.*, suggested Ala 107 and Tyr 110 were spatially close to zinc finger motif based on Mob protein structural view. In addition, there were predicted faced inward to the core of helix bundle, which might contribute to protein stability by hydrophobic interactions (Ye *et al.*, 2009).

3.5 A107G, Y110A point mutate hMOB2 interferes cell spreading

To determine the role of Ala-107 and Tyr-110 of hMOB2 *in vivo*, Ala-107 was mutated to Glycine and Tyr-110 was mutated to Alanine. Protein sequence alignment showed A107G, Y110A point-mutated hMOB2 constructs generated contained histidine-tag and myc-tag sequence (Fig. 13). HT1080 cells were transfected with above point mutated hMOB2 expression constructs, after G418 selection, several colonies of point mutated hMOB2 were selected to generate stable cells lines. We then used Western blot analysis to determine the expression of hMOB2 in these stable cell lines. Results showed

that HT1080 cells transfected with pcDNA-A107G, Y110A-hMOB2 expressed larger size of the recombinant protein than endogenous protein (Fig. 14). The reason is probably due to recombinant protein containing Myc and His tag. We showed that over-expressed hMOB2 promotes its accumulation at the leading edge of HT1080 cells. We then tested whether point-mutated hMOB2 affects its localization at the leading edge. Cells treated with pcDNA-A107G,Y110A-hMOB2 were stained with anti-MOB2, and rhodamine-phalloidin. Immunocytochemistry demonstrated that the hMOB2 expression at the leading edge was suppressed in point-mutated hMOB2. The point-mutated hMOB2 cells expressed hMOB2 expression at the leading edge normally as the untransfected cells (Fig. 15).

To determine the cell spreading in point-mutated hMOB2, cell surface area on images captured at 30 minutes, first hour and second hours after cells replated as showed in the representative photographs (10x) were calculated (Fig. 16A). In point-mutated hMOB2, majority Class 1 cells observed at 30 minutes after cell replating (Fig 16B). Increased with time, at second hour after replating, cells present majority at Class 2. Cells were quantified based on triplicate capture in each experiment repeated in 3 independent experiments. Results were consistent in two point-mutated hMOB2 stable cell lines. These results suggested that point mutation at Alanine 107 to Glycine and Tyrosine 110 to Alanine have affected the promotion of hMOB2 accumulate at the leading edge of HT1080 cells. The quantitative analysis of different treated HT1080 populations at second hours after cells replating showed that down-regulated hMOB2 cells observed majority at Class 1, parent cells at Class 2, over-expressed hMOB2 cells at Class 3 and point-mutated hMOB2 cells at Class 2 (Fig. 16C). Together, these results suggest that

down-regulated hMOB2 delayed cell spreading, over-expressed hMOB2 enhanced cell spreading and point-mutated hMOB2 affected cell spreading by delayed cell spreading.

Actin polymerization actively involved in cell spreading. To study actin morphology, we stained different treated HT1080 population with rhodamine-phalloidin for F-actin during cell spreading. Immunocytochemical results showed extensive membrane ruffles were observed at over-expressed hMOB2 cells compared to parent cells and lesser membrane ruffles were observed in down-regulated hMOB2 cells (Fig. 17). Correlate cell spreading and actin filament at plasma membrane of HT1080 cells, we predicted that hMOB2 participated in cell spreading by affecting actin filament accumulation around plasma membrane. Together, these showed that hMOB2 localized at the leading edge involved in promote cell spreading in HT1080 cells.

3.6 Qualitative changes in overexpression hMOB2 migrating cells

Filopodia and lamellipodia are protrusive structures allowing cells to extend into new area and are commonly found along the cell cortex of spreading cells and at the leading edge of migrating cells (Hall, 1998). We have showed that hMOB2 expression detected at the leading edge of plasma membrane similar to the position of lamellipodia promoted cell spreading in HT1080. Since cell spreading is initial mechanism of cell migration, next, we tested whether hMOB2 participated in cell migration in HT1080 cells using wound healing assays. Confluent monolayers of HT1080 cells expressing endogenous hMOB2, down-regulated, over-expressed or point-mutated hMOB2 were scratched with a pipette tips to induce a wound. The wound edges were monitored over

time by microscopy. The migration rates among the compared cells were quantified by closure percentage. Using this approach we observed similar migration rate in wild type, control, down-regulated, over-expressed and point-mutated hMOB2 in HT1080 cells (Fig. 18). These suggest that hMOB2 expression in HT1080 cells did not affect cell migration and the motility of HT1080 cells is not impacted by hMOB2 expression.

However, immunocytochemistry revealed that rhodamine-phalloidin stained F-actin of the cells bordering injury site showed morphological modifications of the cell migration (Fig. 19). We observed that parent cells seem migrated primarily individually, whereas over-expressed hMOB2 cells migrated in a more collective migration phenotype. This is demonstrated by the unique leading edge, showed by actin cytoskeleton staining. The morphological change induced by expression of hMOB2 was consistent in two individual over-expressed hMOB2 cell lines. In contrast, down-regulated and point-mutated hMOB2 cells appeared to move as individual not in coherent group similar to parent cells, absence of a unique leading edge. This is even more apparent at the higher magnification (Fig. 20). Over-expressed hMOB2 enhanced its accumulation at the leading edge induced broad lamellipodial structures and moved as a coherent group compared with parent cells. In contrast, point-mutated hMOB2 cells did not showed enhancing accumulation of hMOB2 at the leading edge. Both down-regulated and point-mutated hMOB2 cells tend to move as individuals as showed by F-actin stained. Together, these results demonstrate that enhanced accumulation of hMOB2 at the leading edge of HT1080 cells will lead to qualitative changes (collective migration phenotype) as observed in over-expressed hMOB2 cells.

To study whether the cell migration was affected by cell proliferation, hMOB2

effects on the proliferation status of HT1080 cells were investigated. Regardless of initial seeding density, cell growth over a period of 4 days (96 hours) showed no difference between cells in which hMOB2 expression was down-regulated, over-expressed, point-mutated, or wild type cells (Fig. 21). Population doubling times of each cell line calculated using *Doubling Time Software v1.0.10* (Roth, 2006) was between 23 and 25 hours.

4.0 DISCUSSION

How the detached cancer cells from original tumor adhere, attach and spread on new target site is one of the important questions in cancer metastasis. Studies have uncovered numerous genes that participated in cell spreading, but the detailed molecular mechanism of cell spreading process still remains unclear. In this study, we described the *in vitro* functions of hMOB2 in cell spreading in fibrosarcoma cell. We found that cell spreading was delayed in knockdown hMOB2 expression using shRNAs (Fig. 6). Over-expressed hMOB2 in HT1080 cells promoted cell spreading (Fig. 10). Based on these results, we suggest that the hMOB2 protein might play a role in cell spreading.

4.1 Cell spreading Classification

In this study, we quantified the cell spreading stage by categorized cells into three classes. These classifications were set based on Tsumura *et al.* study in year 2005. In Tsumura *et al.* study, cells were categorized into three classes based on the morphology and surface area of cell after replating cells for 30 minutes. Cell present in round and weakly adhered on to the substrate was classified as Class 1, while cell in the course of spreading was classified as Class 2, whereas cell in well-spread with extended pseudopodia was classified as Class 3 (Tsumura *et al.*, 2005). Since different cells have difference morphology and surface area on the differences treated molecular surfaces, however, McGrath explained that the dynamics of cell spreading poses common physical processes despite of the strength of adhesion and the time to complete spreading

(McGrath, 2007). In this study, we determine the surface area of cell in each class based on the morphology explanation in Tsumura *et al*. study. HT1080 fibrosarcoma cell is a highly tumorigenic and poorly differentiated cell. Pseudopodia were observed in early spread cell, thus we defined Class 3 cell based on McGrath, and Senju and Miyata description. Both difference lab's researchers defined that in well-spread cells, the morphology of cells changes became polarized cells with actin polymerized at cell front forming lamellipodia (McGrath, 2007; Senju and Miyata, 2009). Based on the above mentioned morphology, we found that in HT1080 cells, cells pose Class 1 morphology were with cell surface area less than 400μm^2 (Fig. 5). Meanwhile cells pose Class 2 morphology were with cell surface area between 400 μm^2 to 800 μm^2 and Class 3 were with cell surface area more than 800 μm^2. Pseudopodia were observed in Class 1 and Class 2 of HT1080 cells, however, lamellipodia were observed in Class 3 of HT1080 cells.

4.2 hMOB2 functions at the plasma membrane

The function of hMOB2 in cell spreading is currently unclear. Studies have shown that Mob2 in different species have participated in controlling the cell morphology changes by affecting cell polarity and rearrangement of actin cytoskeleton. For example, deletion of mob2 in yeast causes cells to become spherical with depolarized actin and microtubule cytoskeletons affected cell morphology and re-organization of actin cytoskeleton and microtubules (Hou *et al.*, 2003). In mouse Mob2 protein, downregulation of Mob2 in N2A cells resulted in decreased neurite formation, at the

same time, altered the rearrangement of actin cytoskeleton (Lin *et al.*, 2011). During cell spreading process, actin will polymerize at the leading edge of cell membrane, helping the cell to expand, flatten and increase overall surface area. Thus, it will be important to determine whether hMOB2 localization is actin related. In our study, we showed that hMOB2 proteins were localized at the cytoplasm and co-localised with F-actin at the leading edge of plasma membrane when detected using MOB2 antibody (Fig. 2). Although previous study shown that hMOB2 has been detected in nucleus besides cytoplasm and plasma membrane by using various N-terminal tag (Hergovich *et al.*, 2005; Hergovich *et al.*, 2009), in this study, we found no cells expressing hMOB2 in the nucleus when detected hMOB2 expression by using anti-MOB2 antibody. Downregulation of hMOB2 in HT1080 cells affected its localization at the leading edge of plasma membrane, resulting delays in cell spreading (Fig. 4 and 6). In contrast, overexpression of hMOB2 in HT1080 cells promoted its accumulation at the leading edge of plasma membrane, resulting in the enhancements in cell spreading (Fig. 9 and 10). On the other hand, A107G,Y110A point-mutated hMOB2 in HT1080 cells suppressed its localization at the leading edge of plasma membrane and interfered cell spreading (Fig. 15 and 16). Immunocytochemical on different treated HT1080 populations showed extensive membrane ruffles observed at over-expressed hMOB2 cells and lesser membrane ruffles observed in down-regulated hMOB2 cells (Fig. 17). Together, these results suggested that hMOB2 localization at the plasma membrane correlates with actin filament form around plasma membrane and participate in cell spreading.

4.3 Point mutation hMOB2 affects localization of hMOB2

Since regulatory modification of hMOB2 has not been reported thus far, in this study, we try to determine the possible functional domain of hMOB2 participated in cell spreading. Mob proteins shared high sequence similarity and are characteristic of conserved Mob1_phocein domain. In hMOB2 possible functional domain study, we referred the structural-functional domain study on mouse MOB2 (Lin, unpublished data) and the published crystal structure of hMOB1A (Stavridi *et al.*2003). Combining both information, we generated A107G,Y110A point-mutated hMOB2 cell line. We hypothesized that point-mutated hMOB2 will affected its localization at the plasma membrane and affected cell spreading. As expected, the point-mutated hMOB2 cells showed delayed in cell spreading efficacy and decreased expression at leading edge of plasma membrane compared to over-expressed hMOB2 cells. These findings suggest a potential dominant negative role of hMOB2 in HT1080 cells. Here, we provide the first study on regulatory modification of hMOB2. Nevertheless, although studies showed A107G,Y110A point-mutated hMOB2 has potential dominant negative role, studies addressing the involvement of hMOB2 in cell spreading networks have just started. To further support the A107G,Y110A point-mutated hMOB2 as dominant negative in cell spreading, point mutation on others region of hMOB2 is needed to compare with our current point mutation results to rule out the possibility of a mutation artifact.

4.4 hMOB2 does not affect cell migration but affected migrating morphology

Cell spreading is an initial mechanism for cell migration. Both processes involve polymerizing actin at the leading edge formating filopodia and lammelipodia (Flevaris et al., 2007; McGrath, 2007). Hence, cell spreading ability seems to be directly proportional to cell migration. Arthur and Burridge study has shown that integrin-triggered RhoA inhibition by c-Src–dependent activation of p190RhoGAP enhances cell spreading and cell migration by regulating cell protrusion and polarity (Arhur and Burridge, 2001). However, by using the same functional assays to screen different molecular mechanism, Coene and colleagues shown that over-expressed C-terminal of BRCA1 (in common, BRCA1 mutations are associated with an increased susceptibility to breast and ovarian cancer) in Chinese hamster ovary cells (CHO) delay cell spreading, but did not show significant difference in wound healing assay measured cell migration (Coene *et al.*, 2011). Nonetheless, cells exhibit abnormal migration behavior at wound edge in over-expressed C-terminal of BRCA1 in CHO cells. Similar phenomena also seen in Baldassarre *et al.* study, down-regulated filamins in HT1080 cells delay cells spreading and initiation of random cell migration. However, once cell migration has been initiated, down-regulated filamins show compatible migration speed with wild type parent cell (Baldassarre *et al.*, 2009). Together, these suggest that, cell spreading and cell migration are linked process but might not show parallel result. Furthermore, studies mentioned above on cell spreading are linked with actin cytoskeleton (Arhur and Burridge, 2001; Coene *et al.*, 2011; Baldassarre *et al.*, 2009).

Based on the above mentioned, it is able to explain why in our study we observed that hMOB2 affects cell spreading but do not affect motility of HT1080 cells. Interestingly, we also observed qualitative changed in over-expressed hMOB2 cells. In a wound healing assay, over-expressed hMOB2 cells moved as a coherent group, with a unique leading edge and large lamellipodia underlined by actin staining compared with parent cells. Meanwhile, in down-regulated and point-mutated hMOB2 cells, cells moved as a juxtaposition of individuals (Fig. 19 and 20). Fabre-Guillevin and colleagues in study PAI-1 and functional blockade of SNAI1 in breast cancer cell migration show that dominant-negative form of SNAI1 will lead to collective migration phenotype with unique leading edge and numerous large lamellipodia (Fabre-Guillevin *et al.*, 2008). Authors then suggested that this collective phenotype might be due to partial re-expression of E-cadherin detected in dominant-negative SNAI1. Friedl study in collective migration versus single cell migration in cancer cells show that collective migration cells developed preferential integrin and protease with junctions within cells is stabilized by cadherins (Friedl, 2004). Together, these observations suggest that collective migration phenotype observed in over-expressed hMOB2 might be due to the increased expression of cell-cell contact molecules. Thus, an examination of hMOB2 with cell-cell contact molecules will be of interest in the future.

4.5 Partner interacting with hMOB2

Evidence for a direct physical interaction has been reported for Ndr and Mob family members from yeast, flies and mammals (Lerner *et al.*, 2001; Hou *et al.*, 2003; Hergovich *et al.*, 2006; Hergovich *et al.*, 2008; Kohler *et al.*, 2010). Mob2 interacts with

NDR at the N-terminal of SMA (S100/MOB association) domain and trigger the activation of NDR kinase activity (Hergovich *et al.*, 2005; Hergovich et al., 2008). In addition, Hergovich and colleagues mentioned that NDR is recruited by MOB proteins to plasma membrane and lead to full activation of NDR (Hergovich *et al.*, 2005). Thus, it will be worth to further investigate whether NDR interacts with hMOB2 in promoting cell spreading.

In conclusion, our study showed that downregulation of hMOB2 delayed cell spreading and overexpression of hMOB2 promoted cell spreading. We suggest a potential role for hMOB2 participated in cell spreading and regulated by its expression at leading edge of cell membrane. Further research is needed both to pinpoint the relevant protein targets and to identify the signaling pathway involved. We hope our studies will provide further insights into molecular mechanisms of cell spreading.

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Ye, X., Nikolaidis, N., Nei, M., and Lai, Z.C. (2009). Evolution of the *mob* gene family. The Open Cell Signaling Journal *1*, 1-11.

FIGURES

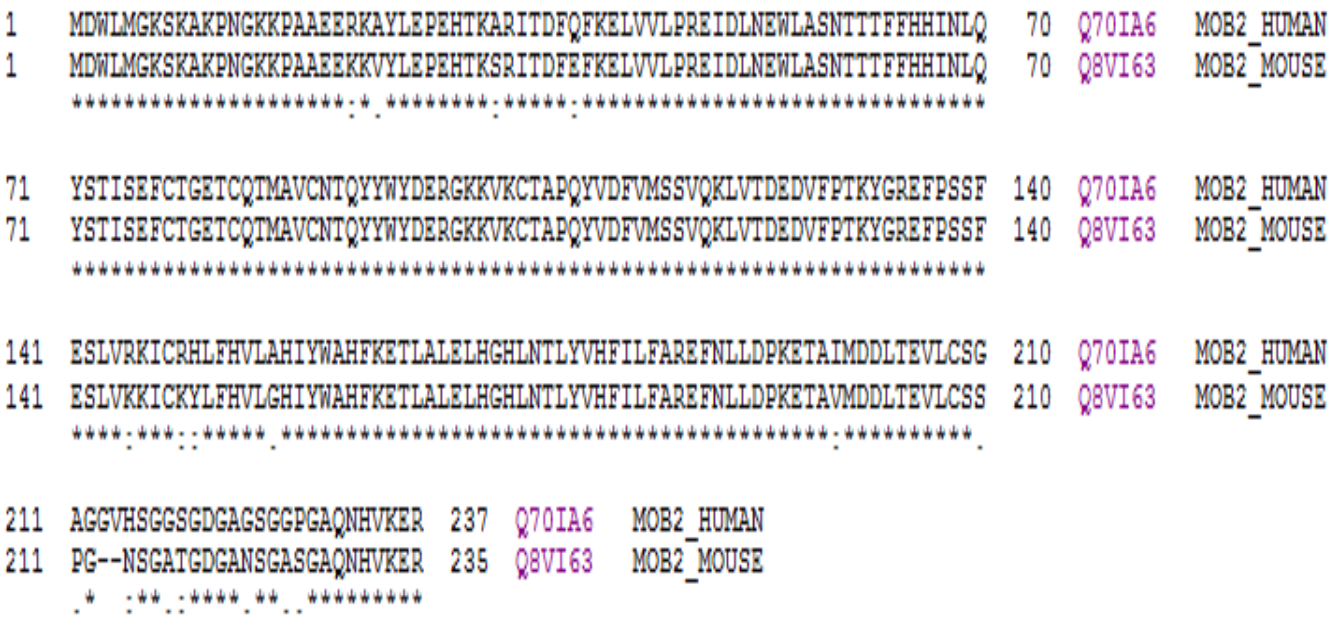


Fig. 1. Sequence alignment of human and mouse Mob2 proteins. Human and mouse Mob2 were highly conserved with 91% identity. The sequences analyzed cover the entire Mob2 length. Dashes are used for alignment gaps. Asterisks are used to locate conserved residues for both aligned sequences. ":" means that conserved substitutions have been observed, whereas, "." means that semi-conserved substitutions are observed.

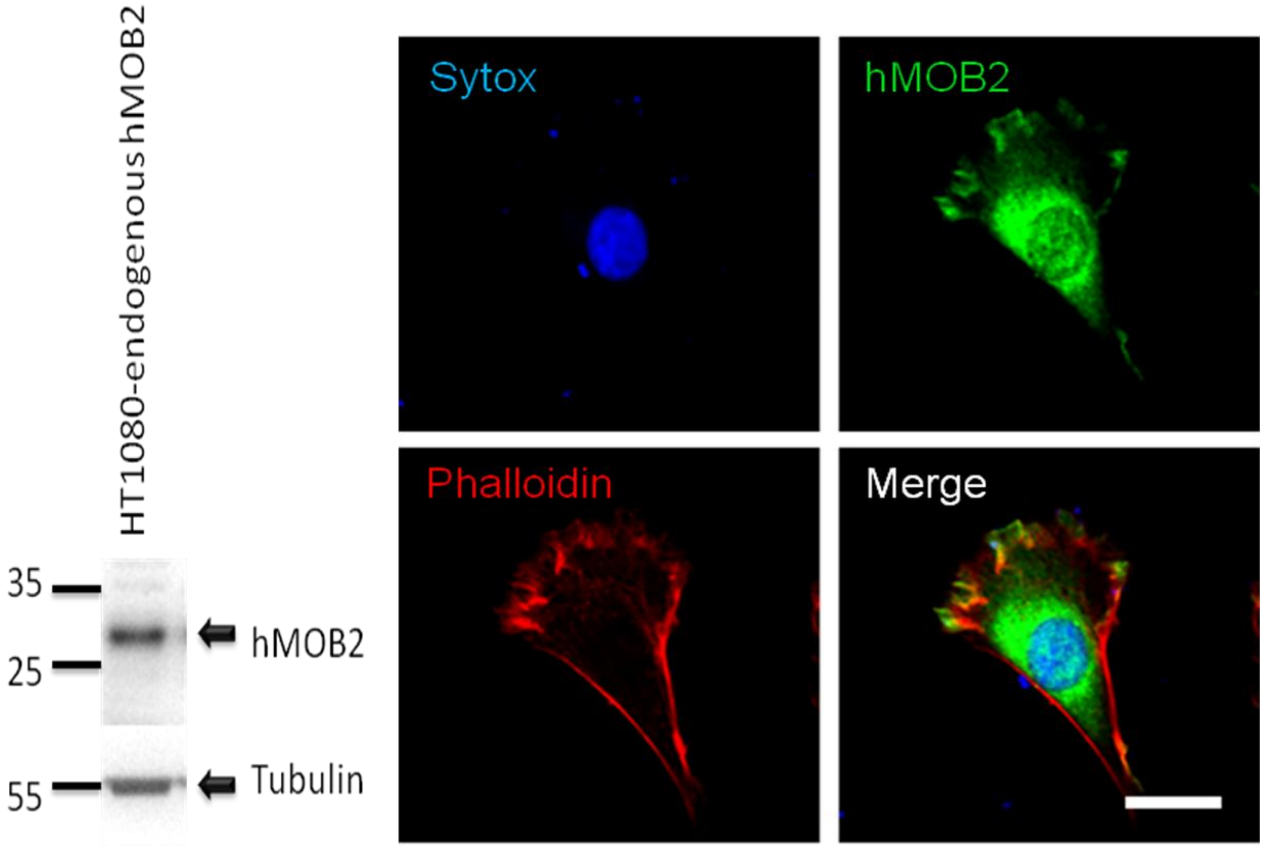


Fig. 2. Subcellular localization of hMOB2 in HT1080 cells. Left: Western blots revealing that anti-Mob2 antibody generated from mouse Mob2 recognizes a 26kDa human MOB2 protein similar to mouse Mob2 protein. Right: Confocal images showed HT1080 cells stained with anti-MOB2 (green), rhodamine-phalloidin (red), and nucleus (blue). hMOB2 expression was concentrated at cytoplasm. Besides that, it also co-localized with rhodamine-phalloidin at the plasma membrane. Scale bar: 20μm.

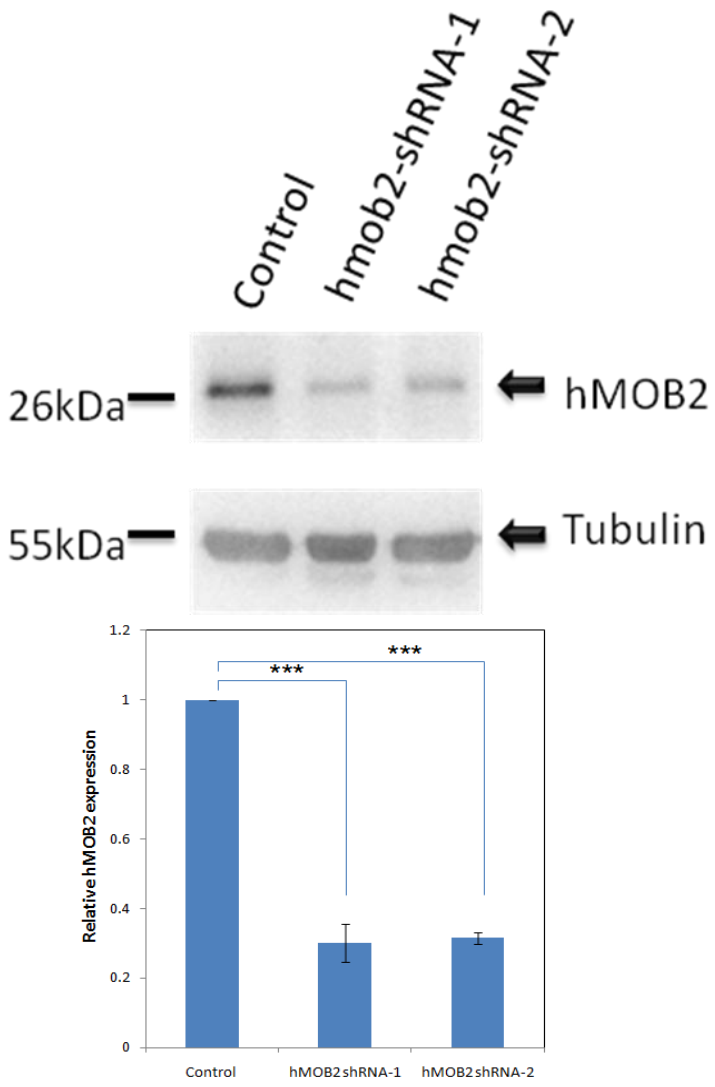


Fig. 3. Downregulation of hMOB2 expression in shRNA treated HT1080 cells. HT1080 cells were infected with control scrambled shRNA and hmob2-shRNAs. Western blot analysis indicated that hMOB2 expression was reduced significantly in hmob2-shRNA treated HT1080 cells. Statistical analysis indicated that the expression of hMOB2 in control and hmob2shRNAs-treated cells were significantly different (three experiments, *Student's t*-test *** indicates $P<0.001$). Tubulin acts as loading control.

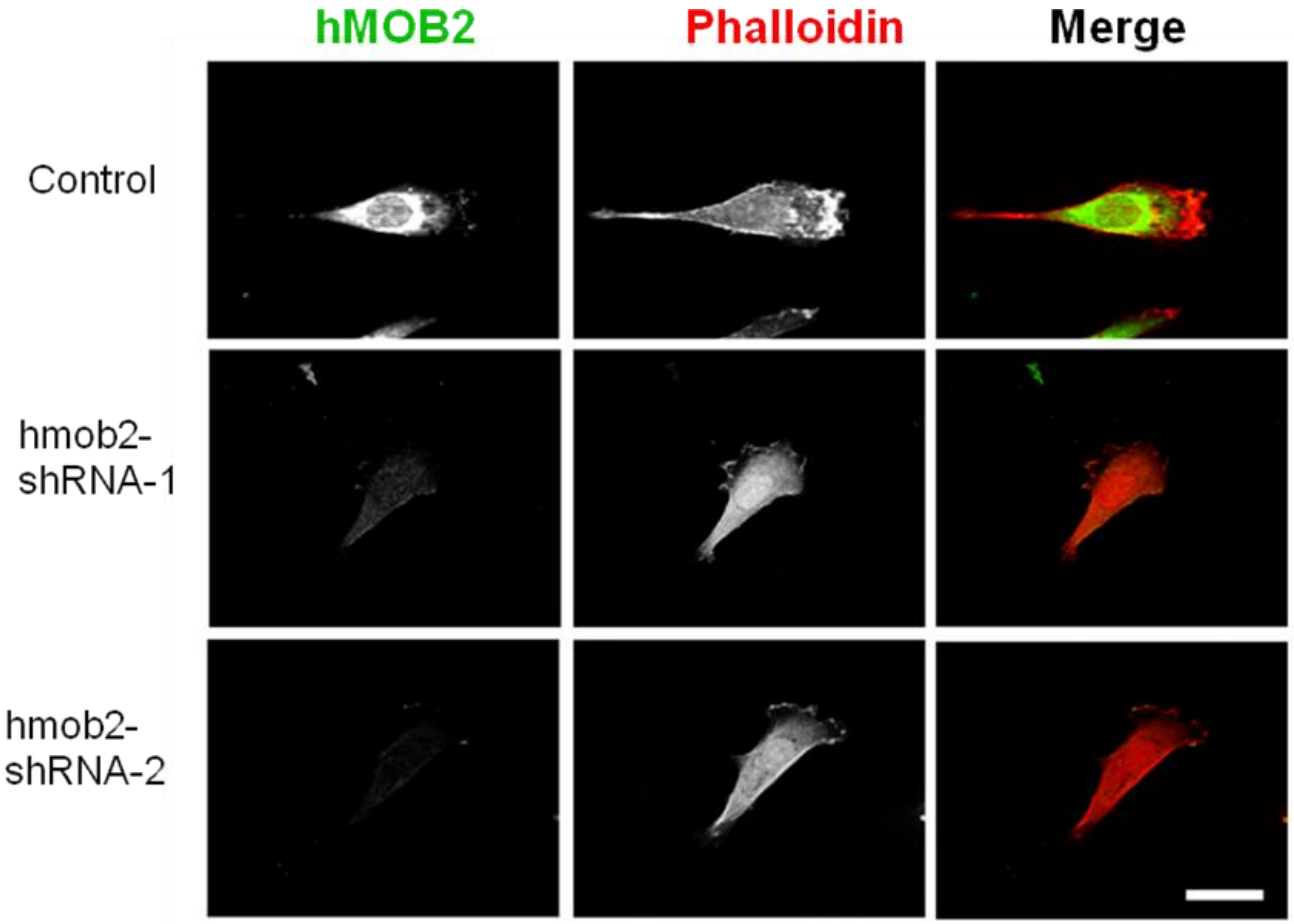


Fig. 4. Downregulation hMob2 does not affect the localization of actin cytoskeleton in HT1080 cells. Parent cells and down-regulated hMOB2 cells were stained with MOB2 antibody (green in merge) and rhodamine-phalloidin (red in merge). In two individual down-regulated hMOB2 cell lines, actin filaments stained by rhodamine-phalloidin occupied the whole cytoplasm with membrane ruffles observed. These observations were similar with endogenous hMOB2 expression. Scale bar: 20μm.

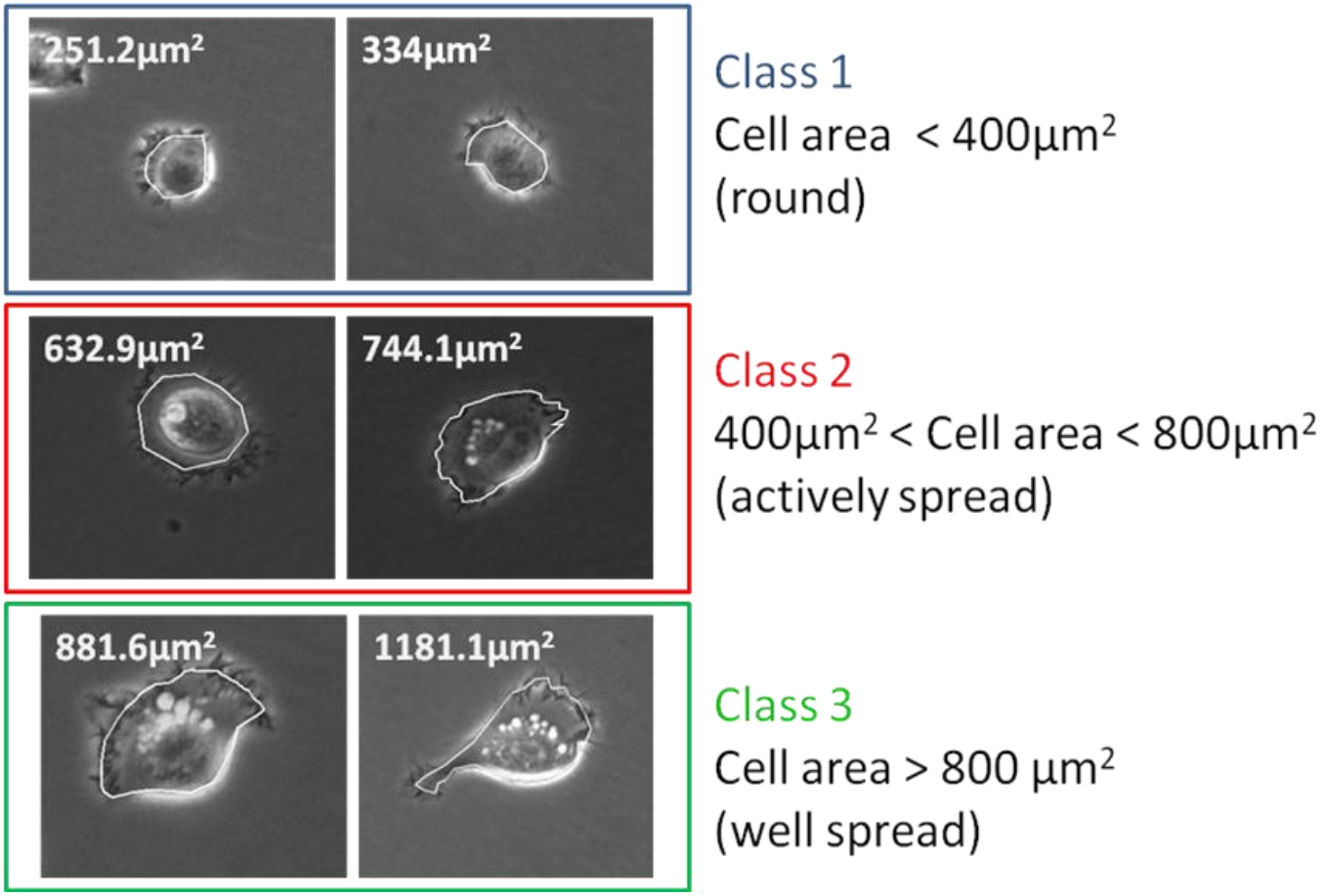
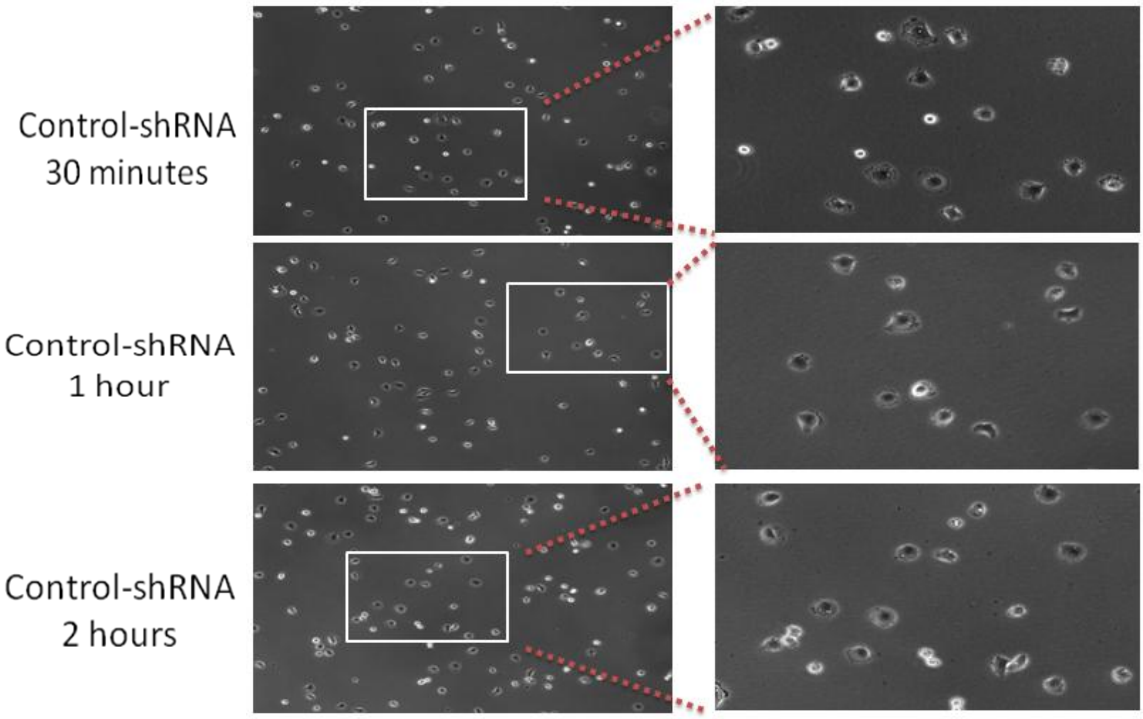
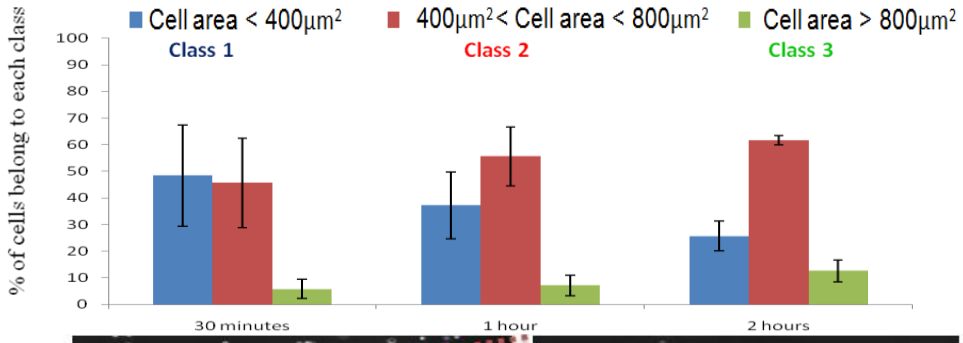


Fig. 5. Three categories of spreading cells. After incubation for 30 minutes, 1 hour and 2 hours, cells images were captured using inverted microscope. The size of cells from 3 random fields for each time and cell line was determined. Cell spreading is characterized by a substantial flattening of the cells and increase in overall surface area. In cell spreading, cells were categorized into three classes; cell area <400 μm^2 (Class 1), 400 μm^2 < cell area < 800 μm^2 (Class 2) and cell area > 800 μm^2 (Class 3).

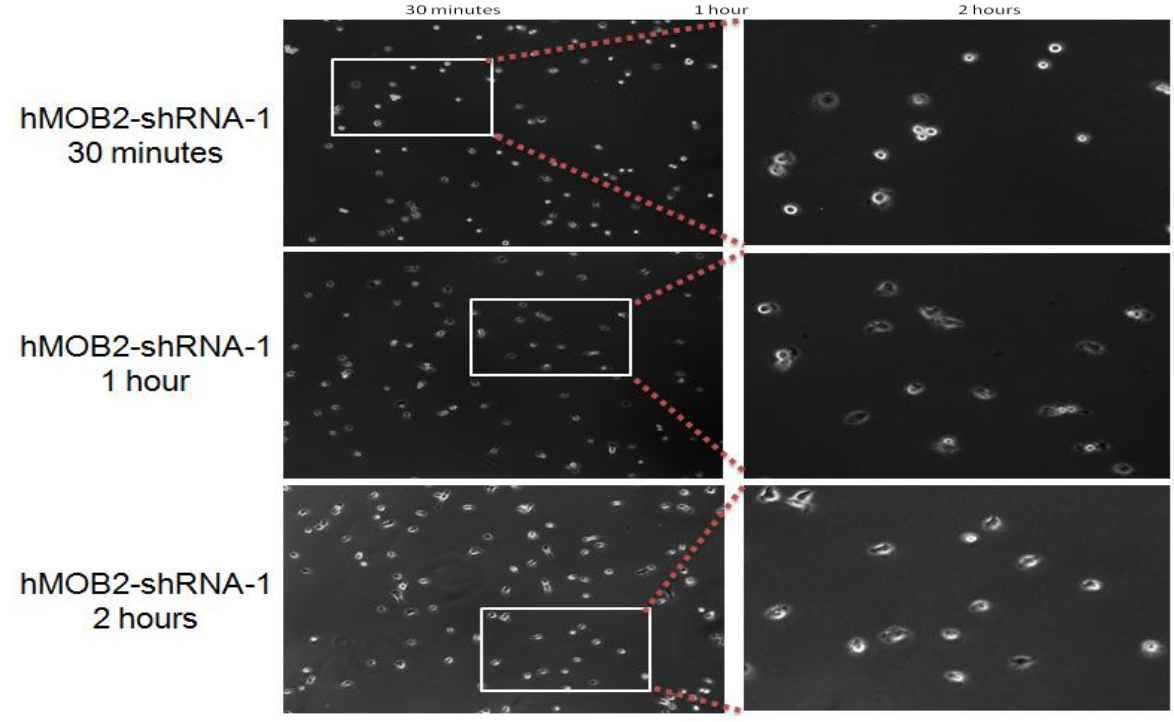
A)


Control-shRNA
30 minutes
Control-shRNA
1 hour
Control-shRNA
2 hours

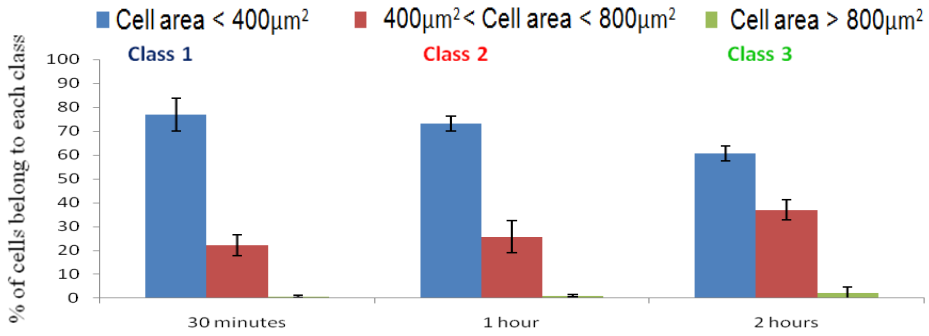
B)


Cell area < 400µm²
Class 1
400µm²< Cell area < 800µm²
Class 2
Cell area > 800µm²
Class 3
% of cells belong to each class
100
90
80
70
60
50
40
30
20
10
0
30 minutes
1 hour
2 hours

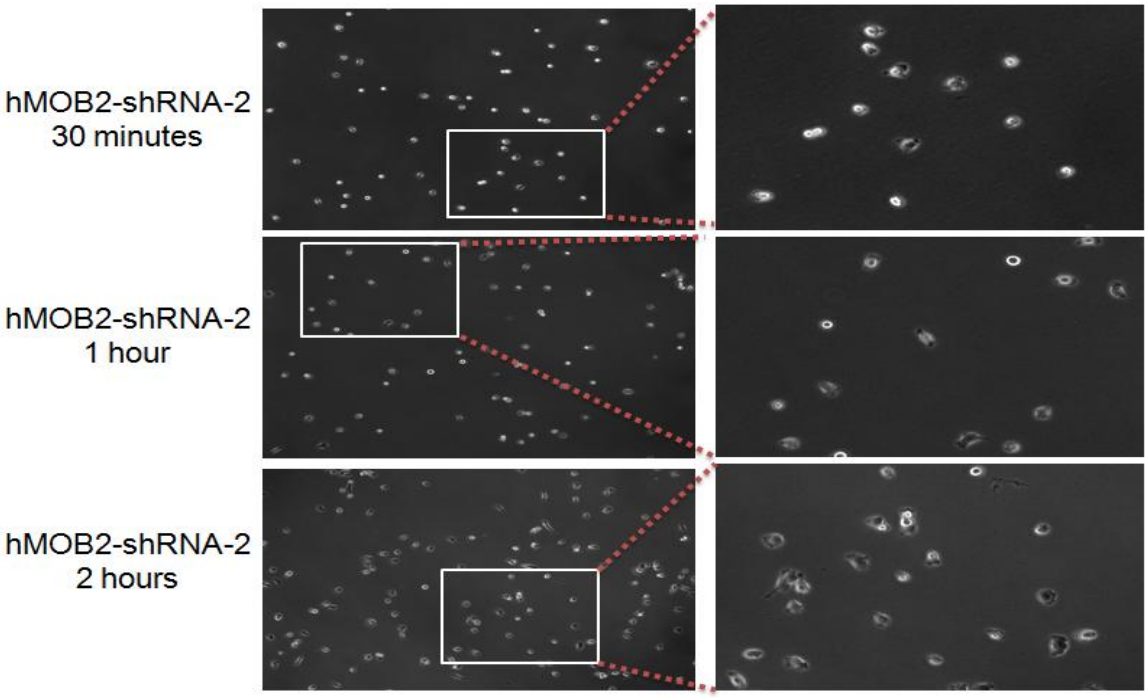
C)


hMOB2-shRNA-1
30 minutes
hMOB2-shRNA-1
1 hour
hMOB2-shRNA-1
2 hours

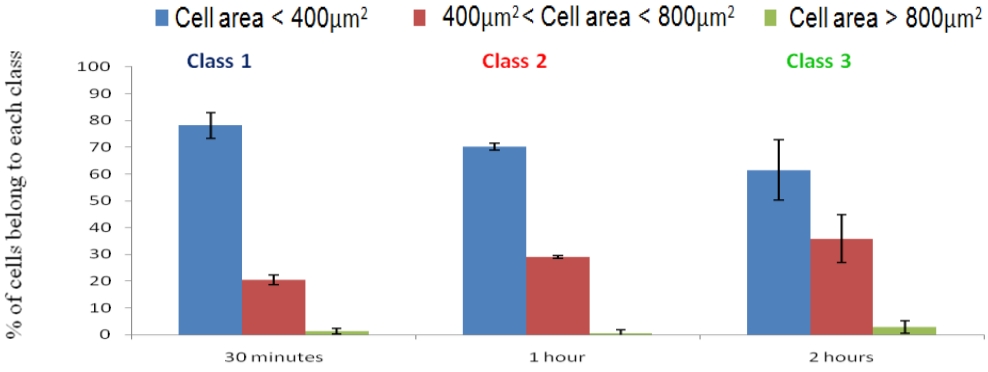
D)


Cell area < 400µm²
400µm²< Cell area < 800µm²
Cell area > 800µm²
Class 1
Class 2
Class 3
% of cells belong to each class
100
90
80
70
60
50
40
30
20
10
0
30 minutes
1 hour
2 hours

E)


hMOB2-shRNA-2
30 minutes
hMOB2-shRNA-2
1 hour
hMOB2-shRNA-2
2 hours

F)


Cell area < 400µm²
400µm²< Cell area < 800µm²
Cell area > 800µm²
Class 1
Class 2
Class 3
% of cells belong to each class
100
90
80
70
60
50
40
30
20
10
0
30 minutes
1 hour
2 hours

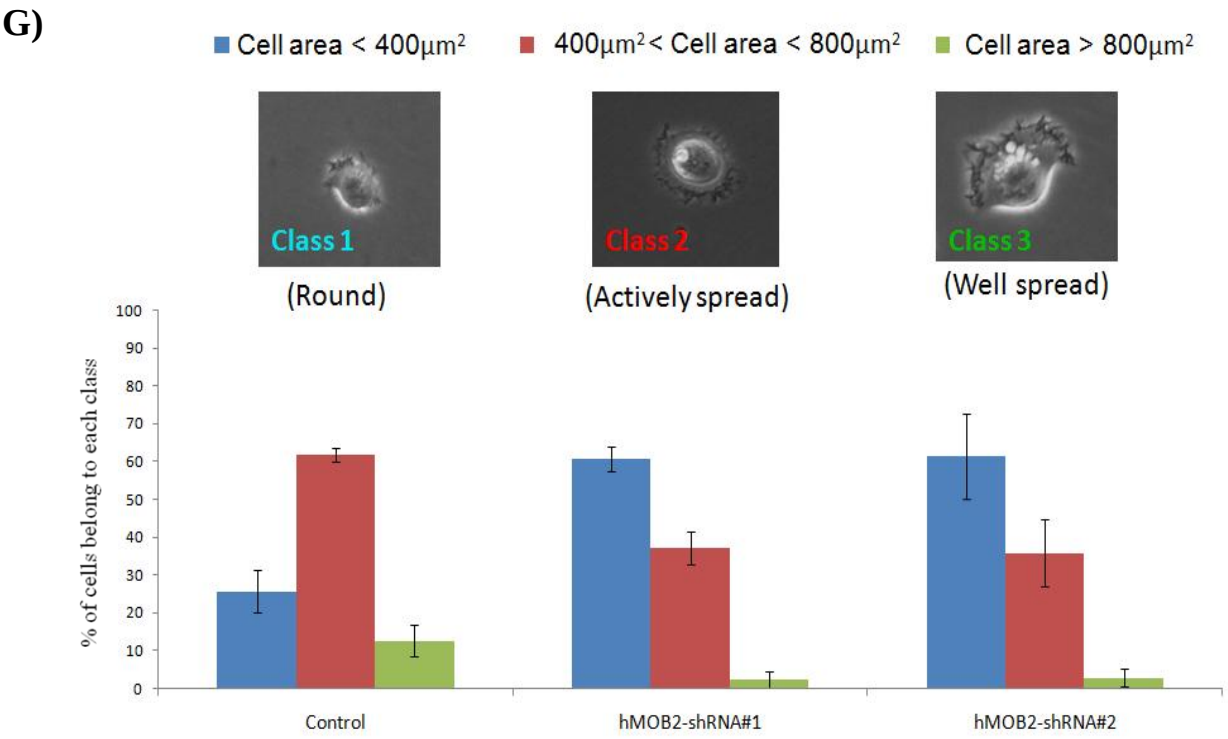


Fig. 6. Downregulation of hMOB2 expression affected cell spreading. Cells suspended and replated, after incubation for 30 minutes, 1 hour and 2 hours, cells images were captured. Cells were classified into three categories (Class 1, Class 2, and Class 3) according to the cell surface area. Photographs were from 1 of triplicate captured in each experiment. At least 180 cells for each group were calculated in each independent experiment. Quantitative analyses were from 3 independent experiments. (A) Representative photographs (10x) at 30 minutes, first hour and second hour after HT1080 cells infected with control scrambled shRNA suspended and replated at 3.5 cm culture dishes. Right photographs represent higher magnification from left indicated regions. (B) Quantitative analysis of the control cell showed Class 1 and Class 2 cells were observed after cells incubated for 30 minutes. Increased with time, at second hour, mainly Class 2 cells observed. (C and E) Representative photographs (10x) at 30 minutes, first hour and

second hour after HT1080 cells infected with hmob2-shRNAs suspended and replated at 3.5 cm culture dishes. Right photographs represent higher magnification from left indicated regions. (D and F) Quantitative analysis of down-regulated hMOB2 cell showed mainly Class 1 cells observed throughout 30 minutes, first hour, and second hour after cells suspended and replated. Results were consistent in 2 shRNA treated HT1080 cells, down-regulated hMOB2 expression. (G) Quantitative analysis indicated that the percentages of different class of cells observed in control and 2 individual down-regulated hMOB2 at second hour after cells replated were different. Class 2, actively spread cells mainly observed in control whereas Class 1, round and weakly attached cells majority observed in down-regulated of hMOB2.

Fig. 7. Protein sequence alignment of generated wild type hMOB2. Protein expression of wild type hMOB2construct generated aligned with predicted hMOB2 protein sequence. Wild type hMOB2 construct generated identical with predicted hMOB2 protein sequence. High consensus portions shown in red are identical amino acids in 2 sequences. Black color underlined shown histidine-tag and myc-tag sequence.

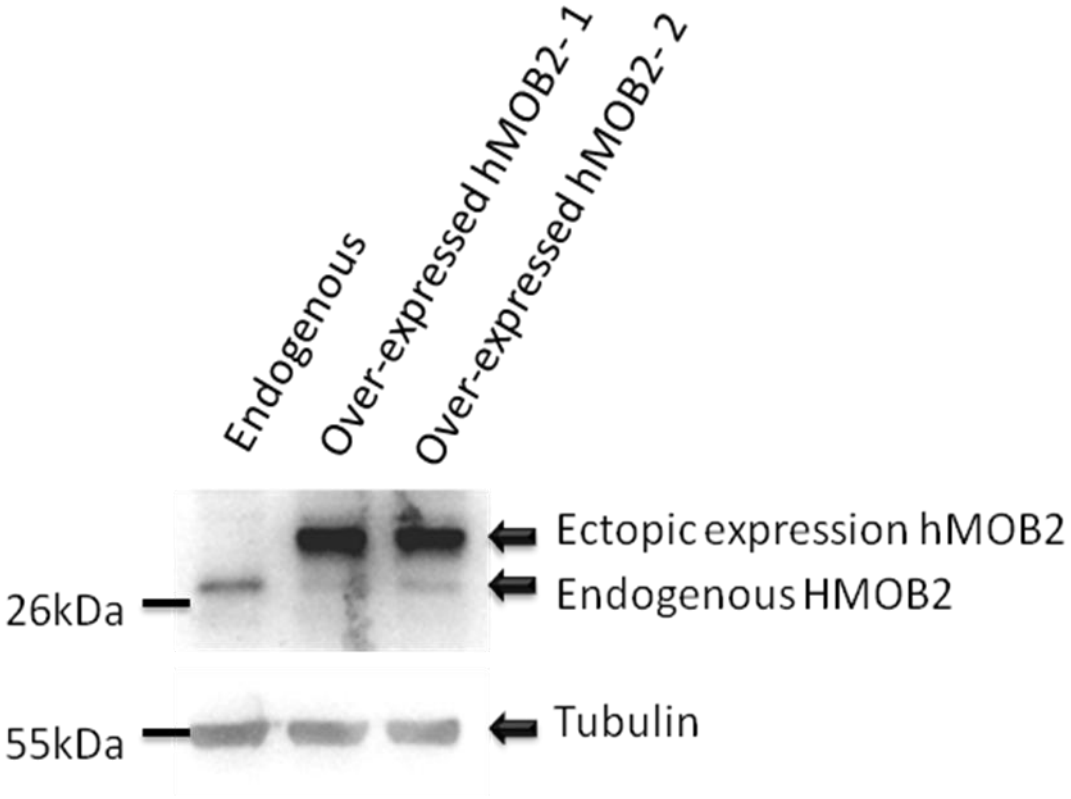


Fig. 8. Overexpression of wild type human MOB2 stable cell lines. Western blot analysis indicated that extrinsic hMOB2 (wild type hMOB2 construct) was introduced and expressed in HT1080 cells. Tubulin acts as loading control.

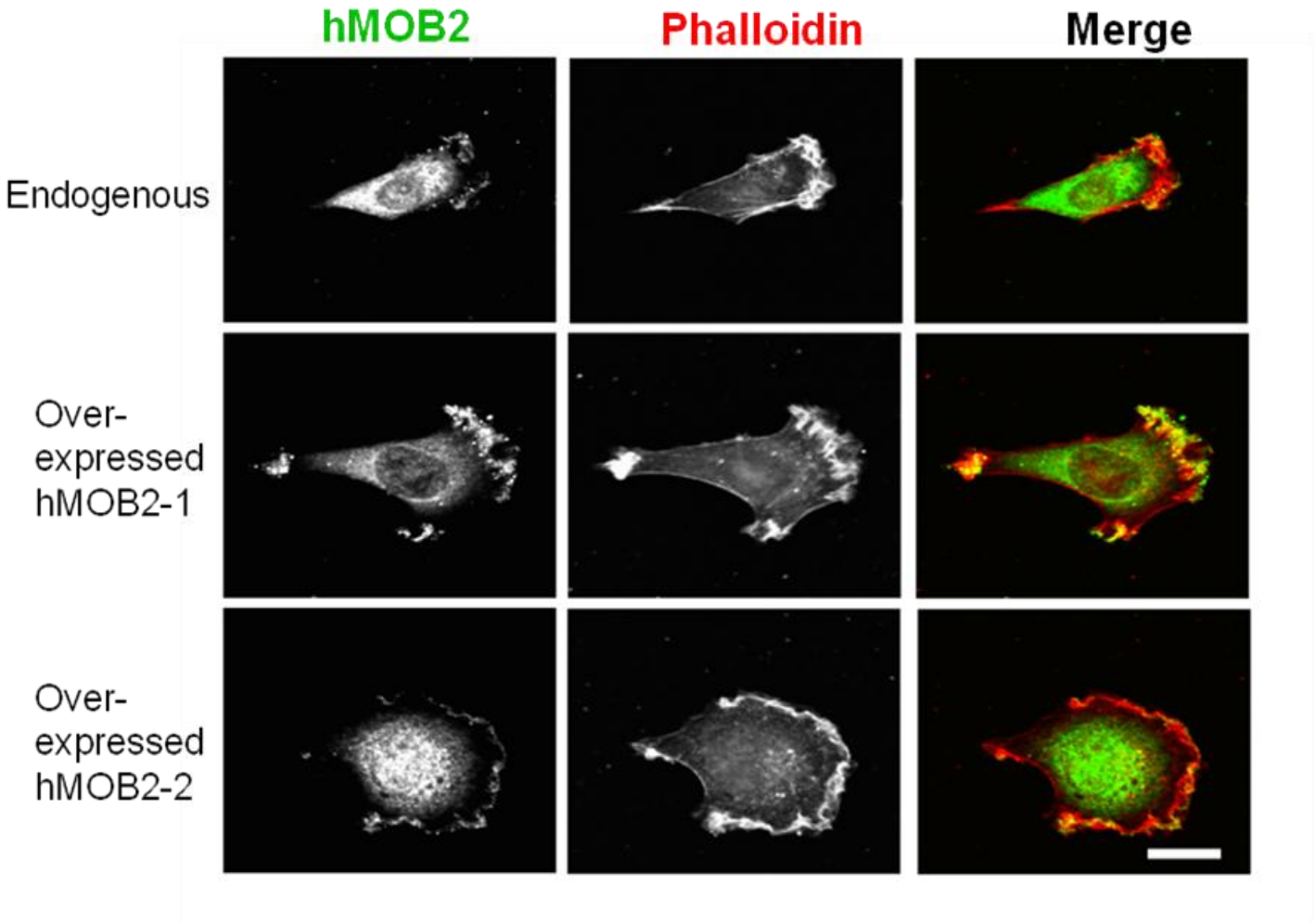
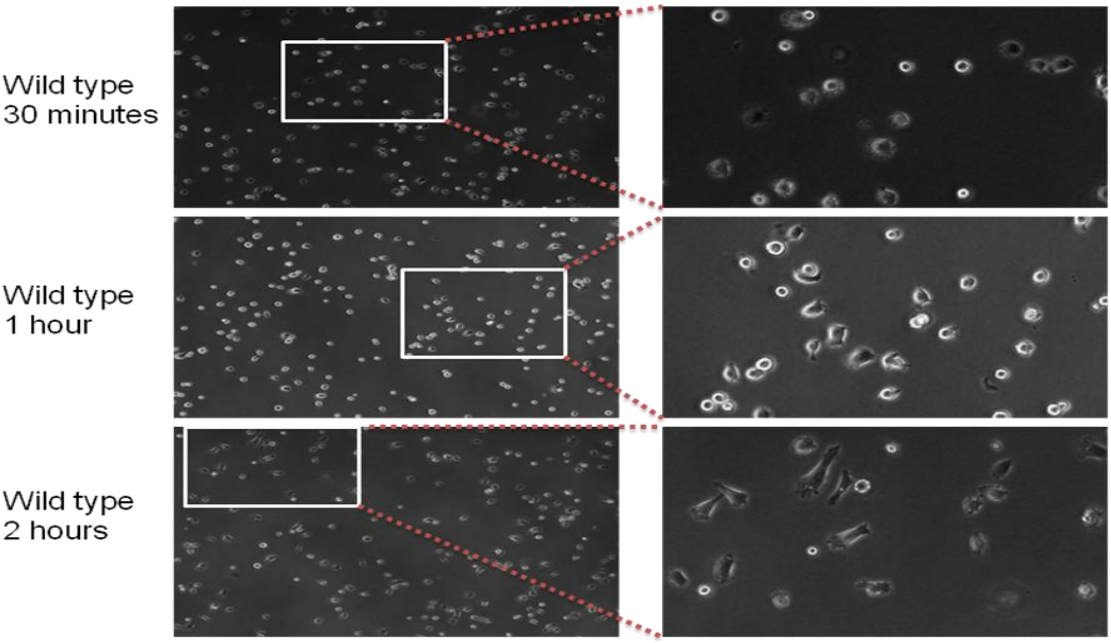
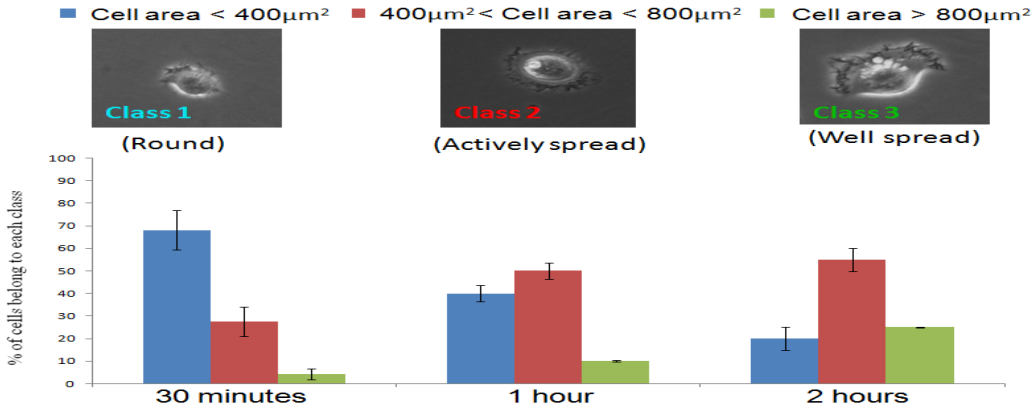


Fig. 9. Overexpression of wild type hMOB2 promoted its accumulation at the leading edge of migrating cells. The parent cells represent endogenous hMOB2 expression and over-expressed hMOB2 cells were stained for anti-MOB2 antibody (green in merge) and for F-actin with rhodamine-phalloidin (red in merge). In parent cells, endogenous hMOB2 expressed normally at the cytoplasm and leading edge of plasma membrane. However, in over-expressed hMOB2 stable cell line 1 and 2, extensive hMOB2 accumulated at the leading edge of its plasma membrane. Scale bar: 20μm.

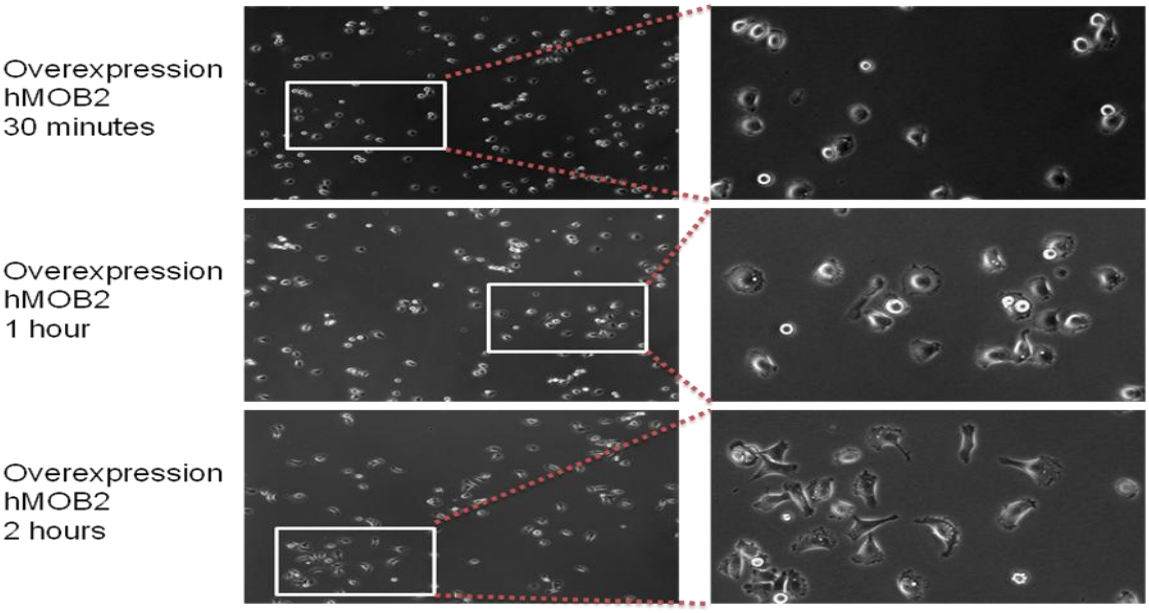
A)


Wild type
30 minutes
Wild type
1 hour
Wild type
2 hours

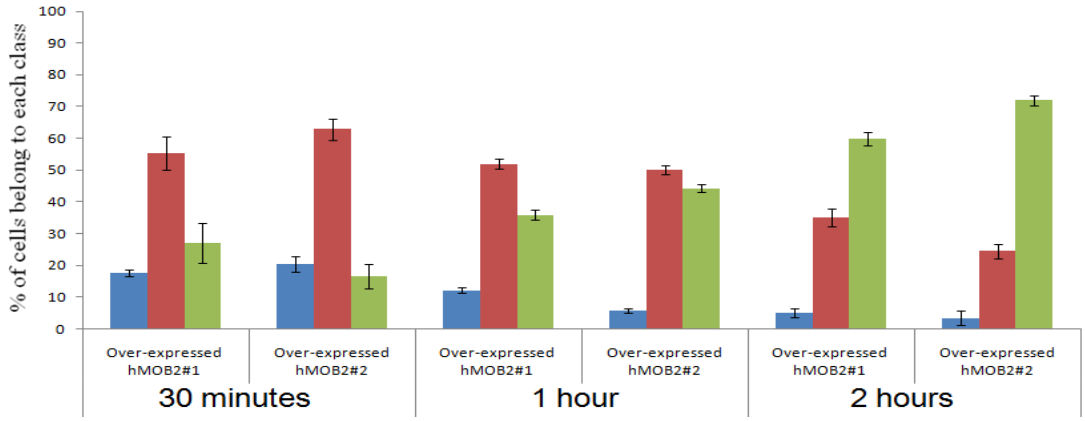
B)


Cell area < 400µm²
400µm² < Cell area < 800µm²
Cell area > 800µm²
Class 1
(Round)
Class 2
(Actively spread)
Class 3
(Well spread)
% of cells belong to each class
100
90
80
70
60
50
40
30
20
10
0
30 minutes
1 hour
2 hours

C)


Overexpression
hMOB2
30 minutes
Overexpression
hMOB2
1 hour
Overexpression
hMOB2
2 hours

D)



E)

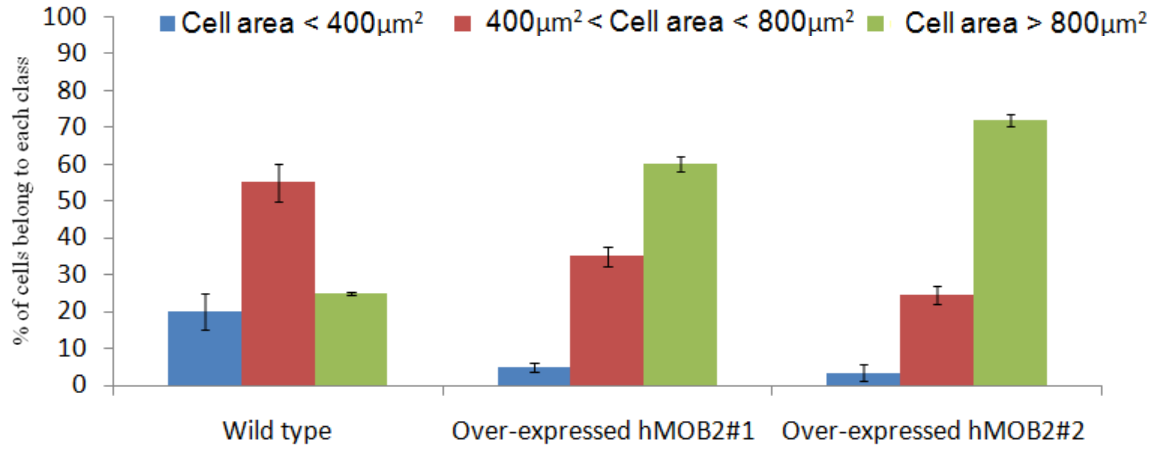


Fig. 10. Overexpression of hMOB2 cells spreads faster than wild type (parent cells). Cells suspended and replated, after incubation for 30 minutes, 1 hour and 2 hours, cells images were captured. Cells were classified into three Classes and percentages of parent cells and over-expressed hMOB2 (at least 180 cells for each group) belongs to each Class were calculated. Results were from triplicate capture in each experiment repeated in 3 independent experiments. Photographs were from 1 of triplicate captured in each experiment. (A) Representative photographs (10x) at 30 minutes, first hour and second hour after wild type HT1080 cells suspended and replated at 3.5 cm culture dishes. Right photographs represent higher magnification from left indicated regions. (B) Quantitative

analysis of the effects of endogenous hMOB2 expression on the spreading of HT1080 cells. Class 1 cells mainly observed after cells incubated for 30 minutes. Increased with time, at second hour, mainly Class 2 cells observed. (C) Representative photographs (10x) at 30 minutes, first hour and second hour after over-expressed hMOB2 in HT1080 cells suspended and replated at 3.5 cm culture dishes. Right photographs represent higher magnification from left indicated regions. (D) Quantitative analysis of the effects of over-expressed hMOB2 spreading in HT1080 cells. Two independent of over-expressed hMOB2 stable cell lines were examined. At 30 minutes, majority Class 2 cells observed. At second hour, cells observed mainly shifted to Class 3. (E) Quantitative analysis indicated that the percentages of different class of cells observed in wild type and 2 individual over-expressed hMOB2 at second hour after cells replated were different. Class 2, actively spread cells mainly observed in wild type whereas Class 3, majority observed in over-expressed of hMOB2. These showed that over-expressed hMOB2 promoted cell spreading.

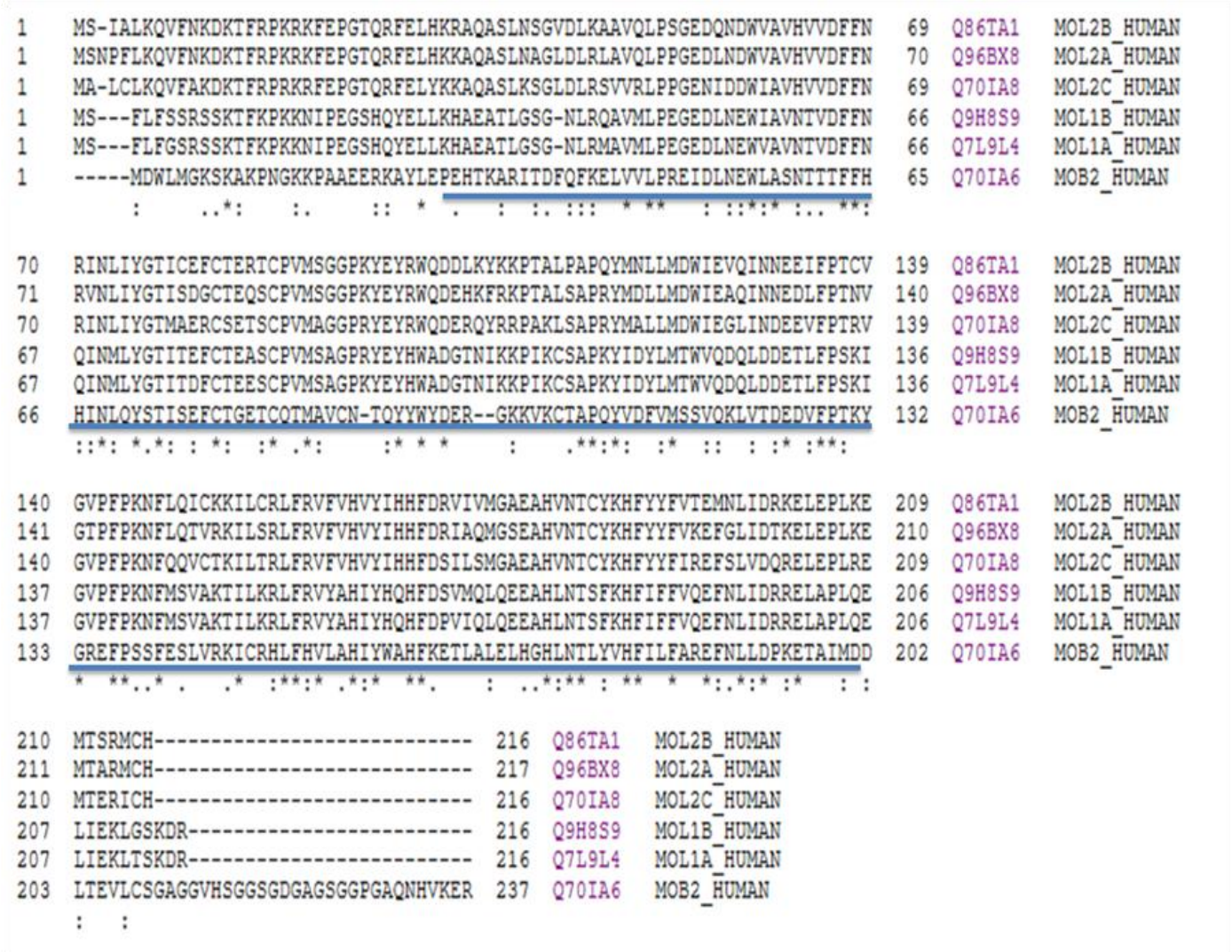


Fig. 11. Sequence alignment of human MOB proteins. Six human MOB proteins were used to make profile alignment covering full length of MOB proteins. Blue line was used to represent Mob1/phocein domain. MOB proteins were characteristic of a conserved Mob1/phocein domain. Dashes are used for alignment gaps. Asterisks are used to locate conserved residues for both aligned sequences. ":" means that conserved substitutions have been observed, whereas, "." means that semi-conserved substitutions are observed.

Fig. 12. Experimental design on point mutation and deletion region of hMOB2 construct. Mouse Mob2 protein possible functional domain region in promoting neurite formation were simulate to human MOB2 protein to determine the possible functional domain of hMOB2 in cell motility. The simulate region contained 116 amino acids. In conjunction with Fig.11, the mentioned important amino acids in human MOB1A within the highly conserved Mob1_phocein domain, several deletion constructs were designed. Blue color rectangular is used to mention the design construct, Ala107 and Tyr110 point mutation hMOB2, was selected in determining the possible functional domain of hMOB2 in cell motility.

Fig. 13. Protein sequence alignment of A107G,Y110A point-mutated hMOB2 construct. Protein expression of A107G,Y110A point-mutated hMOB2 construct generated aligned with predicted hMOB2 protein sequence. Mutant hMOB2 construct generated shown Alanine (blue) at 107 site converted to Glycine (black) and Tyrosine (black) at 110 site converted to Alanine (blue). High consensus portions shown in red are identical amino acids in 2 sequences. Black color underlined shown histidine-tag and myc-tag sequence.

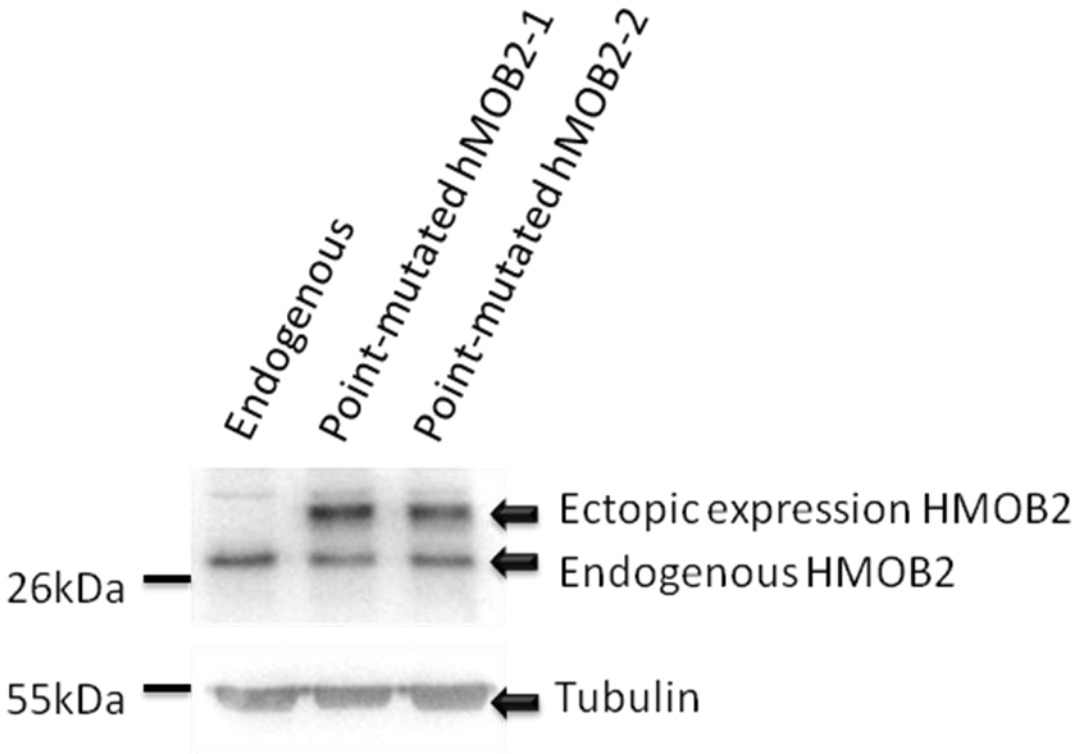


Fig. 14. Overexpression of A107G,Y110A point-mutated hMOB2 stable cell lines. Western blot analysis indicated that extrinsic hMOB2 (A107G,Y110A point-mutated hMOB2 construct) was introduced and expressed in HT1080 cells. Tubulin acts as loading control.

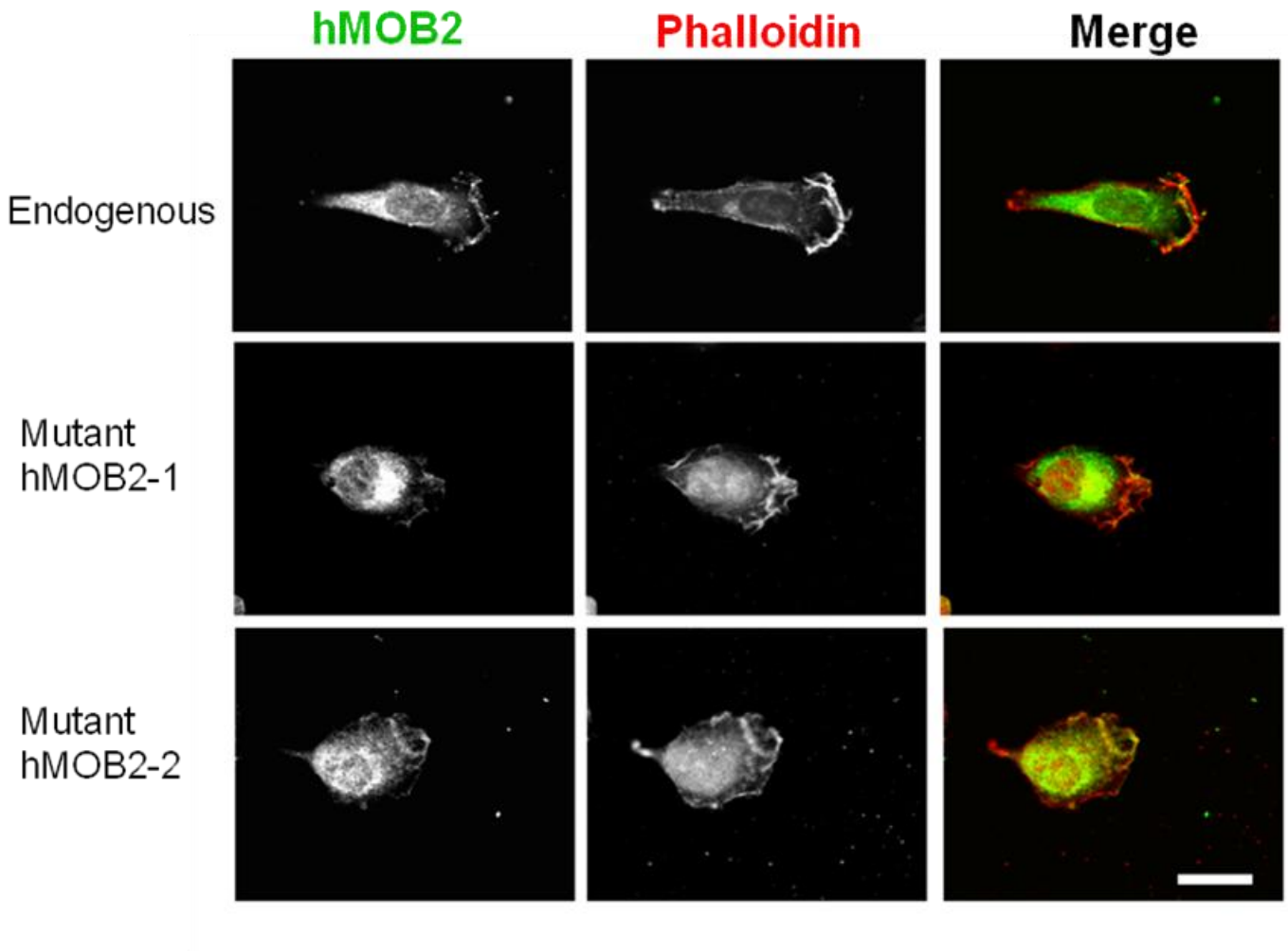
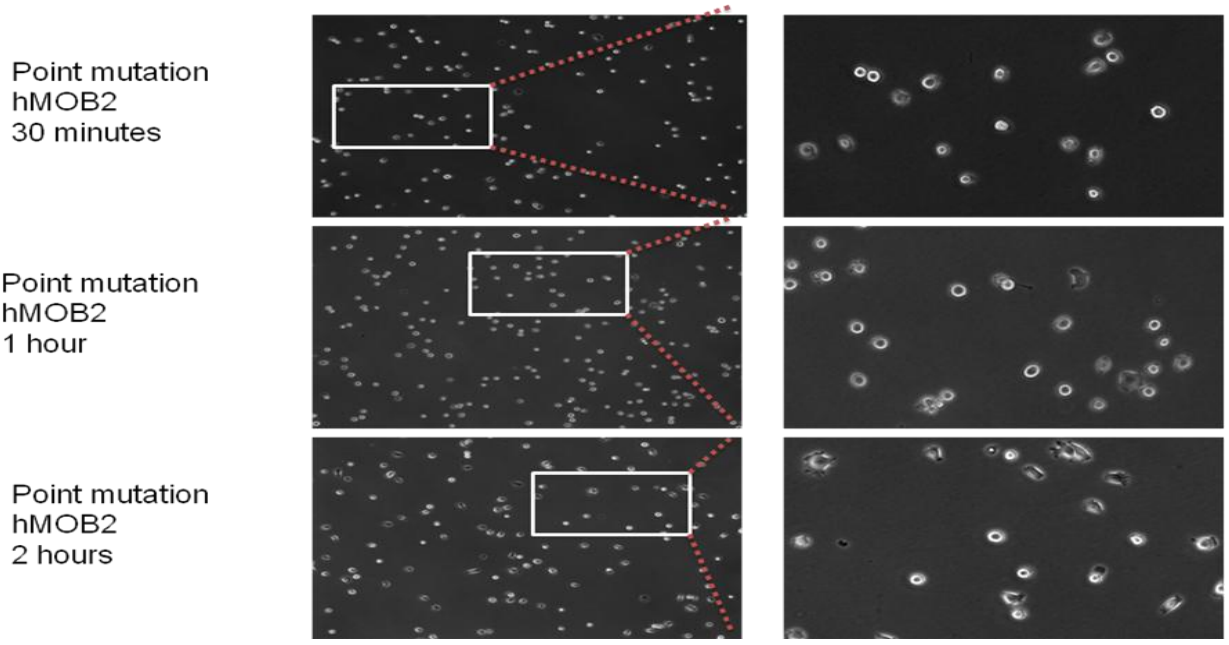
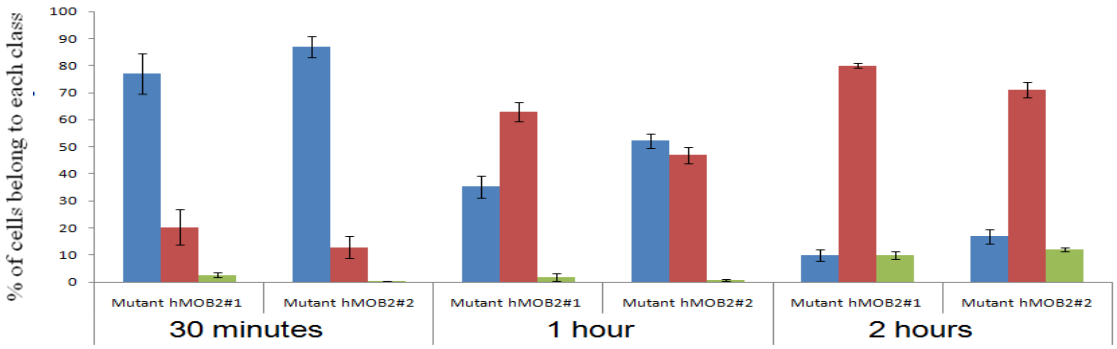


Fig. 15. Expression of A107G,Y110A point-mutated hMOB2 protein suppressed its accumulation at the leading edge. The parent cells represent endogenous hMOB2 expression and A107G,Y110A point-mutated (mutant) hMOB2 cells were stained for anti-MOB2 antibody (green in merge) and for F-actin with rhodamine-phalloidin (red in merge). In parent cells, endogenous hMOB2 expressed normally at the cytoplasm and leading edge of plasma membrane. In addtion, in point-mutated hMOB2 stable cell line 1 and 2, normal hMOB2 expression observed, instead of extensive hMOB2 accumulate at the leading edge of plasma membrane. Scale bar: 20μm.

A)


Point mutation
hMOB2
30 minutes
Point mutation
hMOB2
1 hour
Point mutation
hMOB2
2 hours

B)


% of cells belong to each class
100
90
80
70
60
50
40
30
20
10
0
Mutant hMOB2#1
Mutant hMOB2#2
Mutant hMOB2#1
Mutant hMOB2#2
Mutant hMOB2#1
Mutant hMOB2#2
30 minutes
1 hour
2 hours

C)

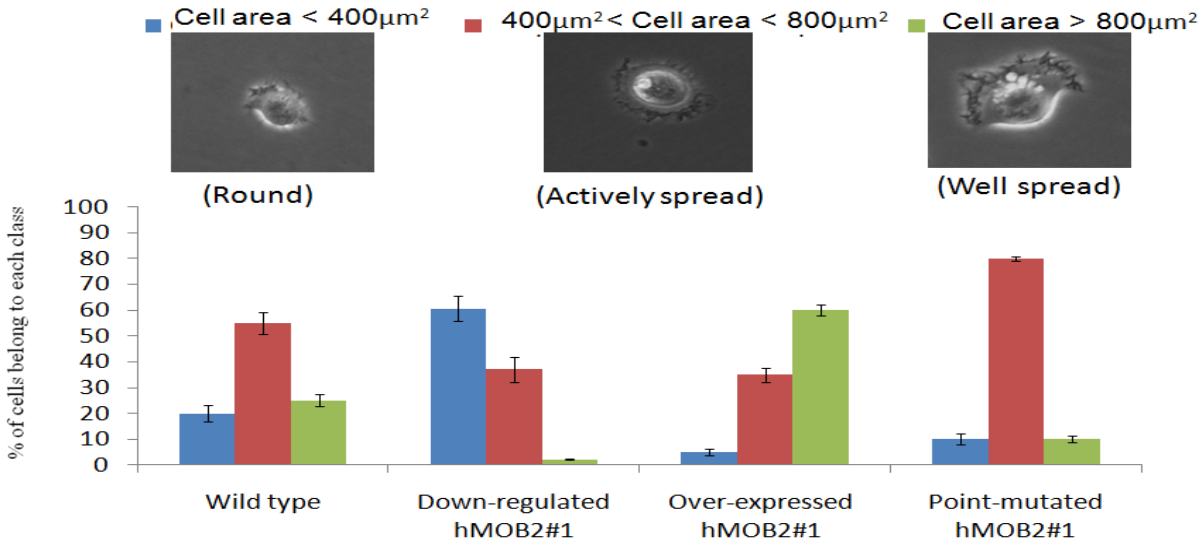

Cell area < 400µm²
400µm² < Cell area < 800µm²
Cell area > 800µm²
(Round)
(Actively spread)
(Well spread)
% of cells belong to each class
100
90
80
70
60
50
40
30
20
10
0
Wild type
Down-regulated hMOB2#1
Over-expressed hMOB2#1
Point-mutated hMOB2#1

Fig. 16. Point-mutated hMOB2 protein interfered cell spreading. Cells suspended and replated, after incubated for 30 minutes, 1 hour and 2 hours, cells images were captured. Cells were classified into three Classes and percentages of parent cells, over-expressed hMOB2 and point-mutated hMOB2 cells (at least 180 cells for each group) belongs to each Class were calculated. Results were from triplicate capture in each experiment repeated in 3 independent experiments. (A) Representative photographs (10x) at 30 minutes, first hour and second hour after point-mutated hMOB2 cells suspended and replated at 3.5 cm culture dishes. Right photographs represent higher magnification from left indicated regions. (B) In analyzed the effects of point-mutated hMOB2 cells in cell spreading, two independent point-mutated hMOB2 stable cell lines were examined. At 30 minutes, cells observed mainly fall in Class 1. At second hour, cells observed mainly at Class 2. (C) Quantitative analysis of wild type, down-regulated, over-expressed and point-mutated hMOB2 cells spreading at second hour after cell replating revealed that over-expressed wild type hMOB2 cells promoted cell spreading, whereas down-regulated hMOB2 cells delayed cell spreading and point-mutated hMOB2 cells affected cell spreading.

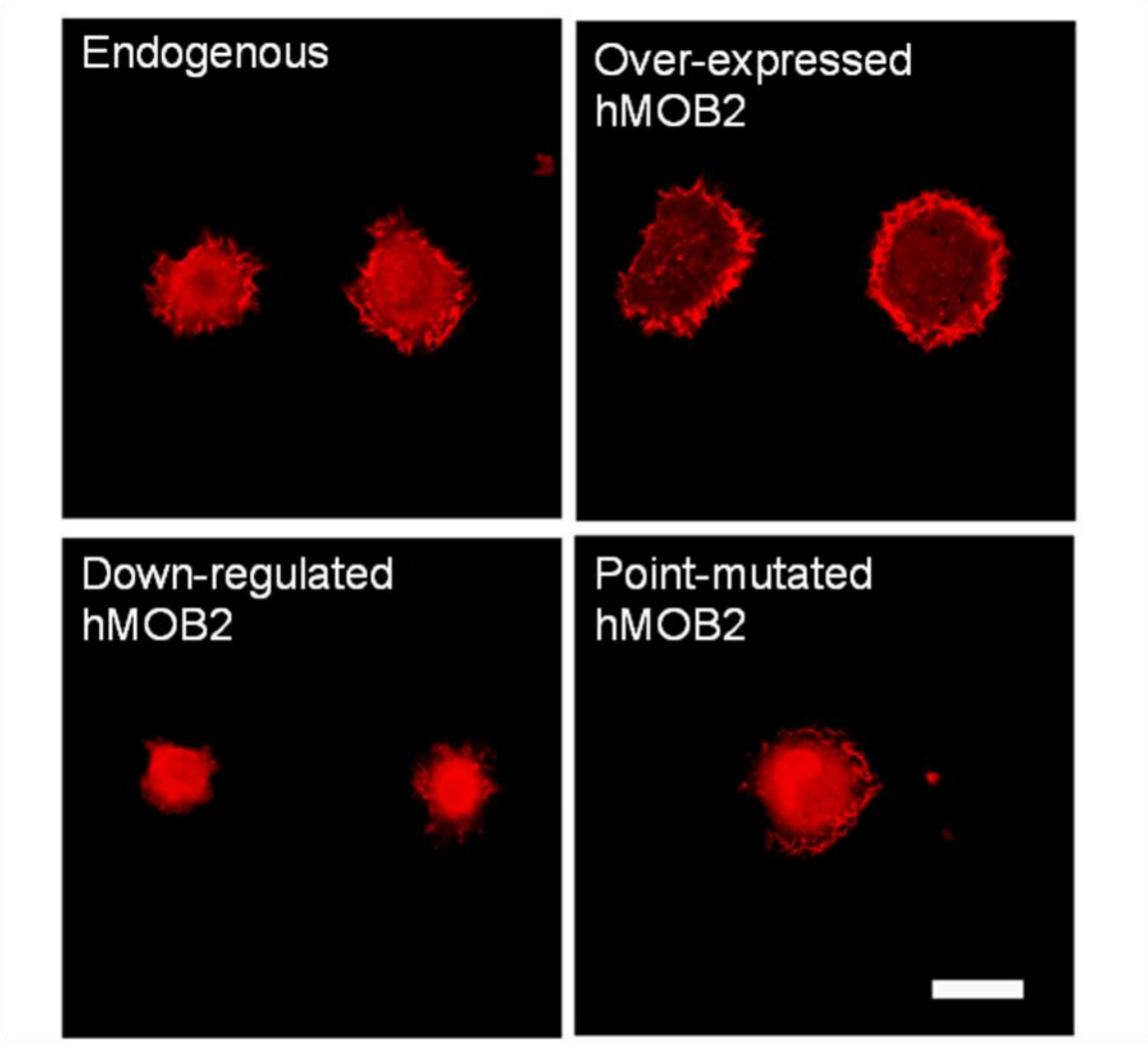


Fig. 17. Actin filament enriched at the plasma membrane of HT1080 cells expressed hMOB2. Difference treated HT1080 populations were allowed to spread on 5μg/cm^2 collagen for 10 minutes before stained with rhodamine-phalloidin. Over-expressed hMOB2 showed extensive membrane ruffles and down-regulated hMOB2 showed lesser membrane ruffles. These suggest that hMOB2 participated in cell spreading by affecting actin filament accumulation around plasma membrane. Scale bar: 20μm.

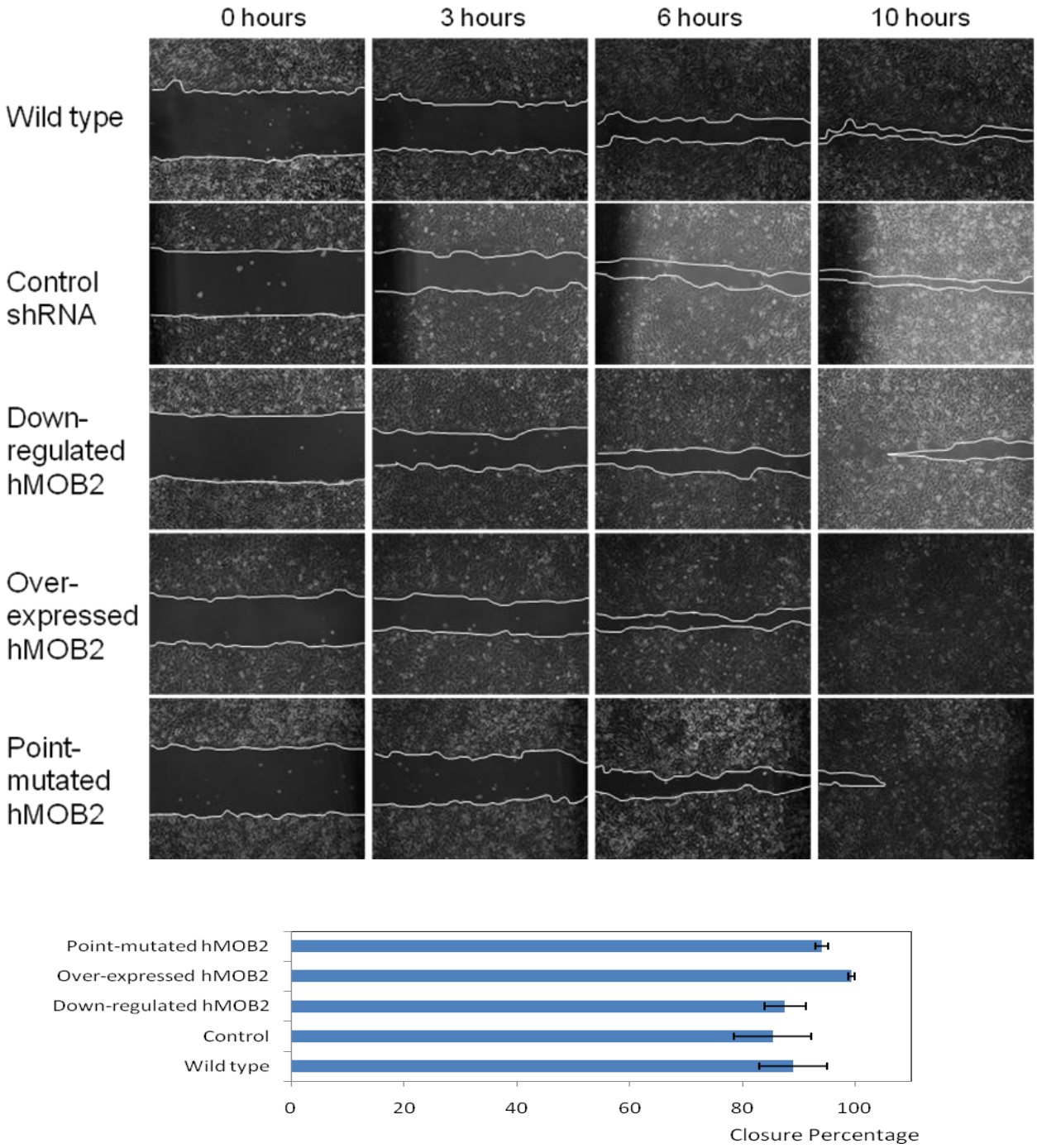


Fig. 18. Wound healing assay shown similar migration rate. Representative photographs (x10) at 0, 3, 6, 10 hours after scrape-wounded cell monolayers from 1 of 3 independent experiments are shown. Degrees of wound closure were assessed by measuring the

overall surface area remains between wound edges after one sample achieved 100% wound closure. Percentages of wound closure are corresponds to the overall surface between wound edges space in each individual experiment normalized to 100% wound closure for that particular sample achieved 100% wound closure in individual experiment. Data are from 3 independent experiments each performed in 2 randomly chosen regions. Over-expressed hMOB2 cells wound closed slightly faster compared to parent cells (wild type). However, it is not significantly different. Similar closure percentages were observed between over-expressed and point-mutated hMOB2 cells. In addition, control treated cells and down-regulated hMOB2 cells also showed similar migration rates when quantified by closure percentage.

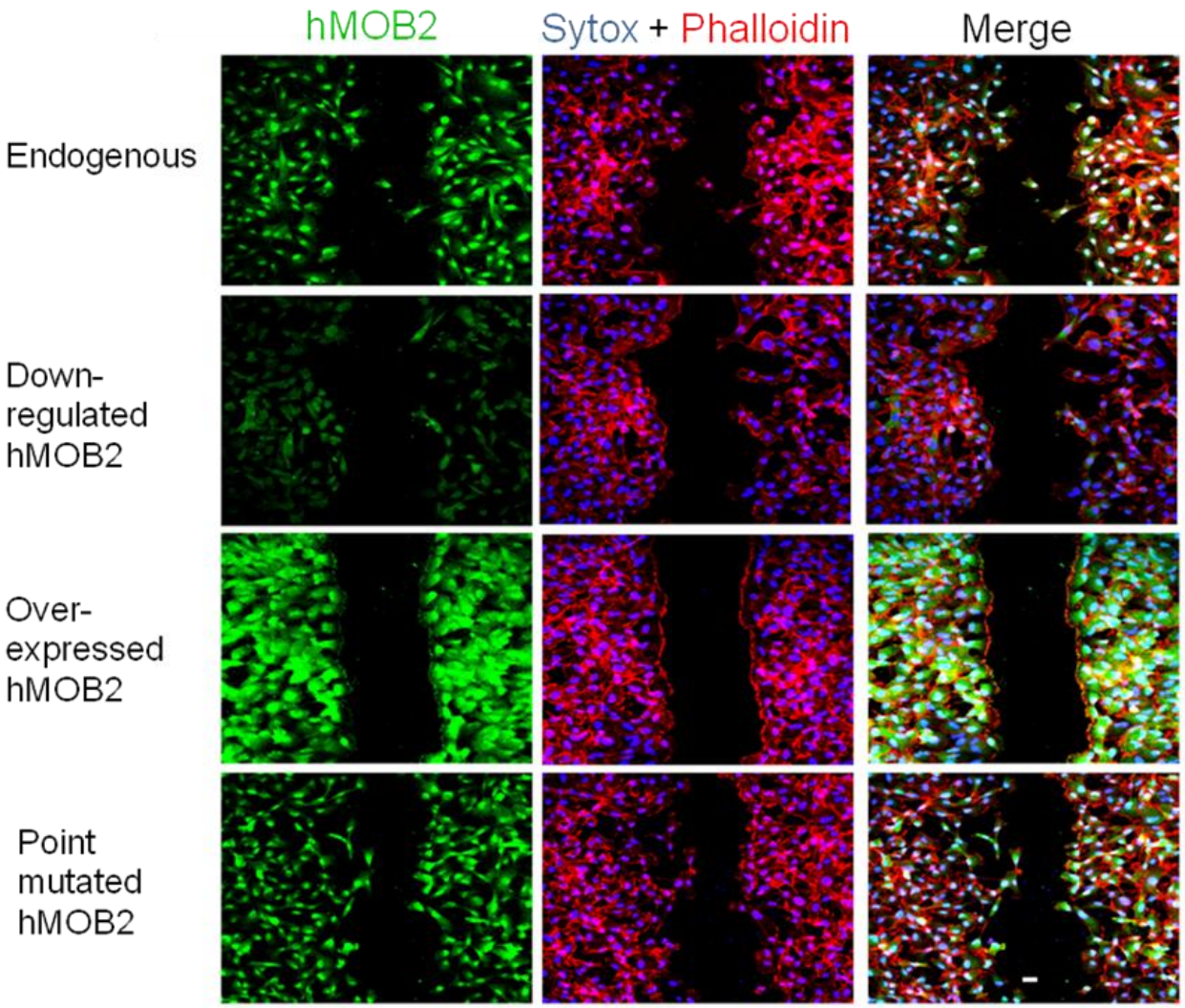


Fig. 19. Qualitative changed in over-expressed hMOB2 cells in cell migration. In a wound healing assay, cells are fixed six hours after wounding. Confocal images showed migrating cells stained with anti-MOB2 (green), rhodamine-phalloidin (red), and nucleus (blue). Rhodamine-phalloidin stained F-actin shown over-expressed hMOB2 cells moved as a coherent group, with a unique leading edge and large lamellipodia underlined by actin staining. In contrast, parent cells down-regulated and point-mutated hMOB2 cells move as individuals. The population is not organized as a coherent group but rather as a juxtaposition of individuals. Scale bar: 20μm.

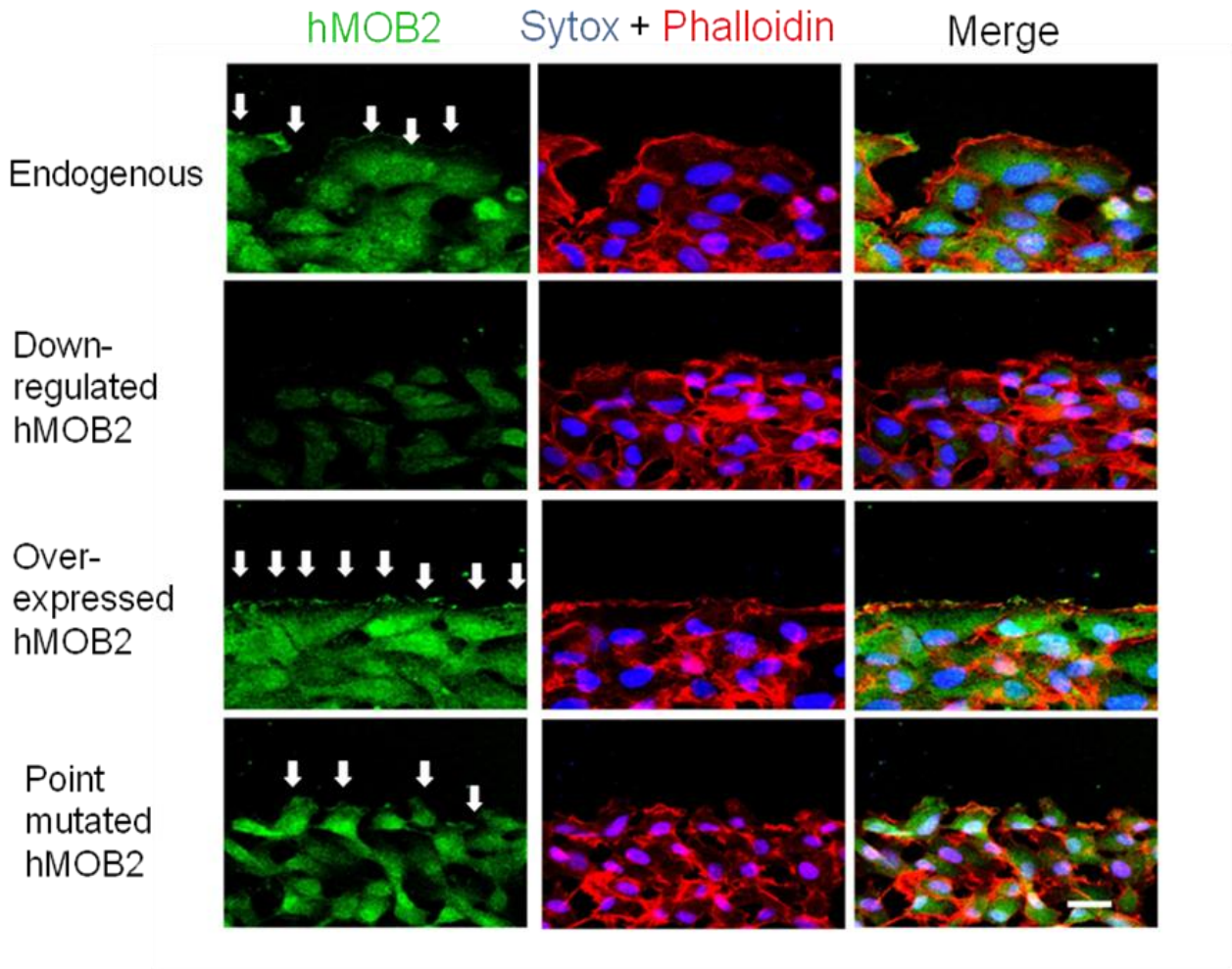
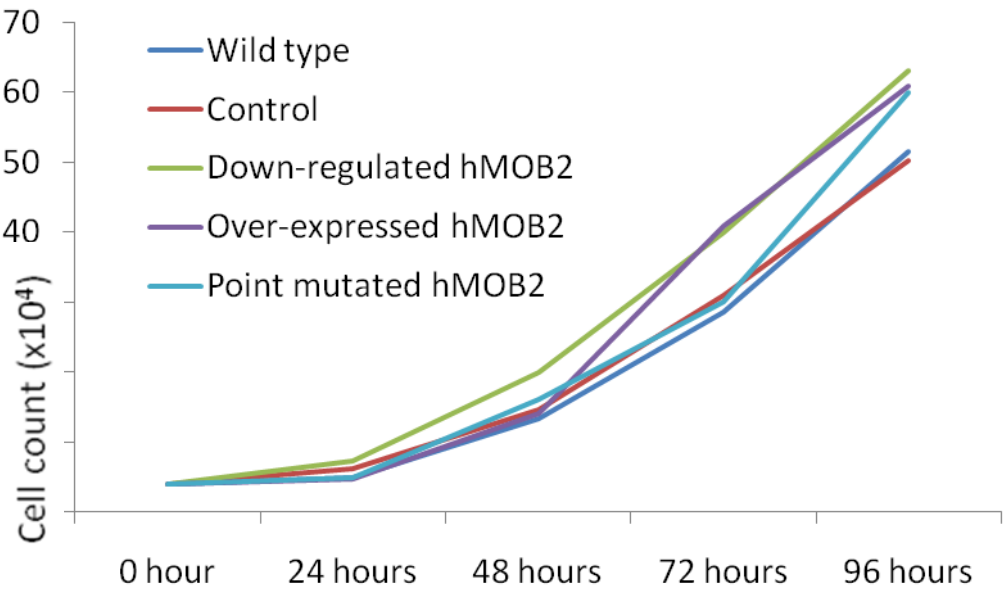


Fig. 20. Higher magnification indicated qualitative changes in over-expressed hMOB2 cells in wound healing assay. Cells are fixed six hours after wounding. Confocal images showed migrating cells stained with anti-MOB2 (green), rhodamine-phalloidin (red), and nucleus (blue). Arrows indicate hMOB2 at the leading edge co-localized with rhodamine-phalloidin stained F-actin. Over-expressed hMOB2 enhanced its accumulation at the leading edge induced large lamellipodia, moved as a coherent group compared with parent cells. In contrast, point-mutated hMOB2 cells did not showed enhancing accumulation of hMOB2 at the leading edge. Both down-regulated and point-mutated hMOB2 cells tend to move as individuals as showed by F-actin stained. Scale bar: 20μm.



	Doubling time
Wild type	24.22
Control	25.82
Down-regulated hMOB2	25.37
Over-expressed hMOB2	23.15
Point mutated hMOB2	23.33

Fig. 21. Cell proliferation assay. Cells were plated at a density of 40000 cells/well in a 24 wells. After cells culture for 24 hours, 48 hours, 72 hours and 96 hours, cell growth was measured directly by counting suspended typsinized cells using Hemocytometer. Experiments were performed in triplicate. Quantitative analysis showed similar proliferation rate observed between HT1080 parent cells (wild type), control, down-regulated, over-expressed and point-mutated hMOB2 in HT1080 cells. Population doubling times of each cell line was calculated using *Doubling Time Software v1.0.10* (Roth, 2006) and indicated at the table below the graph.